QUANTITATIVE AND MECHANISTIC ANALYSIS OF MAMMALIAN CELL CULTIVATION ON MICROCARRIERS

by

Wei-Shou Hu

B. S. National Taiwan University
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Signature of Author _____
Department of Nutrition and Food Science
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Certified by _____
Thesis supervisor

Accepted by _____
Chairman, Committee on Graduate Students,

This thesis has been examined by a committee of the Department of Nutrition and Food Science as follows:

Prof. Arnold L. Demain _

Chairman

Prof. Daniel I. C. Wang _

Thesis Supervisor

Prof. Lan-Bo Chen _____

Prof. Robert S. Langer

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ABSTRACT

Many mammalian cells used for the production of biochemicals are anchorage-dependent. They require the attachment to a compatible surface in order to grow. The use of microcarriers enables the application of stirred tank reactors to the cultivation of anchorage-dependent mammalian cells as well as the maintenance of high ratios of growth surface area to reactor volume during scale-up. However, in the past, several problems have impeded the large-scale operation of microcarrier culture. In this thesis these problems are examined quantitatively using human foreskin fibroblasts, FS-4, cultivated on microcarriers.

For the cultivation of mammalian cells on microcarriers, a minimum inoculum concentration is required to initiate a successful culture. Both the growth rate and the growth extent decrease as the inoculum concentration is reduced. A critical cell number model is presented to elucidate the mechanism of the inoculum requirement. In this model it is proposed that after inoculation a critical number of cells per microcarrier is required for normal growth to occur, otherwise cell growth is impeded. This critical cell number model is expressed mathematically and used to simulate cell distribution and growth on microcarriers under different cultivation conditions. By comparing the simulated growth kinetics with experimental results, the critical cell number per microcarrier was identified. The critical number could be reduced by employing an improved medium for cultivation. Additionally, in order to reduce the inoculum requirement, the microcarrier diameter can be selected rationally. Applying the results of these analyses, the inoculum requirement for FS-4 cells was reduced four- to

five-fold. This gave rise to a sixteen-fold increase in cell number during batch cultivation.

For the large-scale operation of microcarrier culture to be successful, a technically feasible inoculation method to seed subsequent microcarrier cultures is essential. This has been achieved by detaching cells from microcarriers using a high pH trypsinization technique. The detached cells reattach to new microcarriers at a higher rate than to used microcarriers on which cells have previously grown. The effect of this differential cell attachment was analyzed and overcome by employing a low inoculum concentration. Cells could thus be serially propagated on microcarriers and subsequently used for product formation. A method of optimizing the microcarrier diameter for serial propagation of mammalian cells on microcarriers at a low inoculum concentration is presented.

On other engineering aspects, oxygen transfer in microcarrier culture systems was examined. The rate of oxygen transfer by surface aeration was improved by more than five-fold with the use of a surface aerator. The effect of shear force exerted by agitation on the growth of FS-4 cells was also studied. Excessive agitation resulted in a decrease in the growth extent. An integrated shear factor was found to correlate with the growth extent under different agitation conditions. However, under the agitation conditions used for microcarrier culture, shear force is not likely to cause a deleterous effect on cell growth in large-scale operation.

Thesis Supervisor: Dr. Daniel I. C. Wang

Title: Professor of Chemical Engineering

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INTRODUCTION

The development of animal cell cultivation *in vitro* is far more recent than that of microbial fermentation. The use of perforated cellophane and glass particles for cell cultivation by Earle *et al.* (1951) some thirty years ago marked the first development toward large-scale cultivation of animal cells. Since then animal cell culture technology has steadily developed. It is now widely used in the production of many industrially important biologicals including vaccines, monoclonal antibodies and interferons. Animal cell culture technology owes much of its progress to the discovery of antibiotics. By suppressing the growth of contaminating microorganisms, antibiotics have made much of the research in cell cultivation possible. Both animal cell culture and antibiotic fermentation were in their infant stage in the early fifties. While antibiotic fermentation has become a major biochemical process, and its operation a subject of numerous scientific and engineering studies, our understanding of the animal cell culture from a engineering point of view is still limited.

Many mammalian cells, used for the production of biologicals, are anchorage-dependent. They need to attach to a compatible surface in order to grow. Conventionally, anchorage-dependent cells are grown on Petri dishes or roller bottles which are not suitable for large-scale processes. To contain a large growth surface area in a single bioreactor

microcarriers were developed by van Wezel (1967). This development also allowed stirred tank reactors to be used for anchorage-dependent cell culture. A subsequent development of microcarriers of DEAE-Sephadex with a low ion exchange capacity by Levine et al. (1979), allowed cells to grow on microcarriers to a high density. However, some problems have impeded the application of microcarrier cultures to large scale processes. Some of these problems will be addressed in this thesis.

Most animal cell cultivation is practiced in a batch fashion. Cells are cultivated on a compatible surface to confluence and then detached from the surface and reinoculated onto a larger surface area for further multiplication. Two of the major problems, related to the large-scale operation of microcarrier cultures, are the requirement for a large inoculum and the inability to initiate a culture with microcarrier grown cells.

For microcarrier culture a relatively large inoculum concentration is required to initiate a batch culture. Failure to supply sufficient inoculum results in an impeded growth rate and a reduced growth extent. For normal diploid fibroblasts, such as FS-4, the inoculum size is often as high as 30 to 40%. Since only a few fold increase in cell concentration can be achieved in each propagation step, a large number of multiplication stages is required to to reach the production scale. It is desirable to reduce the inoculum cell concentration to make microcarrier culture an industrially economical process.

Cells grown on conventional surfaces, e.g. Petri dishes or roller bottles, can be detached by treatment with proteolytic agent(s) along with mild mechanical pertubation. These cells are then used as the inoculum of another culture. However, a technically feasible method for the detachment of microcarrier grown cells has not been demonstrated, and thus these cells cannot be used to inoculate another culture. Instead, cells grown in roller bottles are required for the inoculation of a microcarrier culture. This problem has been compounded by the large inoculum concentration required to achieve desirable growth rates and growth extents. The large number of roller bottles required for the inoculation of a single microcarrier culture makes inoculum preparation a prohibitive task, especially in large scale operations. If the use of microcarriers for anchorage-dependent cells is to be technically feasible for large scale operation, a method of direct inoculation from a seed microcarrier culture is essential.

The application of microcarriers to cell culture is a relatively recent development. Because of this, until now the research has been of a largely exploratory nature. Unlike other areas of biotechnology, in which various optimization strategies have been developed, most of the operation parameters in mammalian cell cultivation have not been examined from the reaction engineering point of view.

The main goal of this thesis is to analyze the above two problems of microcarrier culture from a quantitative and mechanistic point of view. The objective is not only to shed

light on the solutions to those problems, but also to demonstrate that reaction engineering principles can be applied to mammalian cell culture technology. Two other important engineering parameters, oxygen transfer and the effect of shear force on cell growth were also examined.

LITERATURE SURVEY

Animal cell culture has long played an important role in biotechnology. Many valuable biologicals such as vaccines and interferons are produced by cell culture. However, the mainstream of biotechnology has always been, and will continue to be, microbial fermentation. To a large number of scientists or engineers in biotechnology, the characteristics of animal cells in culture are often unfamiliar. The first half of this literature survey is therefore devoted to a short introduction to biological aspects of cell growth *in vitro.* The second half deals with recent progress in cell cultivation on microcarriers.

I. CELL GROWTH IN VITRO

Animal cells are often categorized according to their anchorage-dependency. Some cells can grow in suspension, such as lymphocytes, while others such as fibroblasts, epithelial, endothelial and epidermal cells must attach to a compatible surface in order to grow. Lastly, there are cells which can grow either in suspension or anchored to a surface. Most cells in this third category are transformed and are derived from anchorage-dependent cell types, such as HeLa.

The first report on the serial propagation of cells dates back to nearly seventy years ago. Using a trypsin solution, Rous and Jones (1916) were able to disperse cells from tissue and grow them in a clot. They subsequently redispersed the cells from the clot and were able to obtain subsequent growth.

Normal cells exhibit a variety of social behaviors. After cell attachment, the cells will spread and become elongated in shape and align with each other in a parallel, polarized fashion. Abercrombie and Heaysman (1954) showed that both cell movement and multiplication are inhibited at high cell density. Using time-lapse photography, they showed that cells cease movement when they collide with each other. After contact is established, they do not overlap each other. Thus, a monolayer of cells is formed when the growth surface is completely covered with cells. Cells from many different tissues can be cultured <u>in vitro</u> and subjected to serial passage. However, for normal diploid cells, the life-span is finite. They have only limited replicative potential in culture. In a study using normal human fibroblast cells, Hayflick and Moorhead (1961) observed that the final fate of those cells was death. After explanted from tissues, cells grew actively for a number of passages but all eventually reached a period of poor growth called crisis. Cells died subsequent to crisis. For cells derived from human embryonic tissues, proliferation is usually sustained for 40 to 70 population doublings. During the period of active growth, a very large increase in cell number can be attained. For example, ten million cells can be obtained from an embryonic tissue without

much difficulty. Fourty population doublings would give rise to a total of 10^{19} cells or a cell mass equivalent to millions of people. The immense cell number potentially attainable is abundant for any practical use (Hayflick and Moorhead, 1961).

All normal fibroblasts are thought to be mortal, although the number of precrisis passages may vary (Hayflick, 1977). In the case of human fibroblast cells, death follows the crisis. For cells of other species, the outcome subsequent to crisis can be different. Rodent cells can survive crisis to give rise to permanent or established cell lines. However, these cell lines are different from those of a normal tissue. During the period of active growth, normal fibroblast cells do not show a significant deviation from the normal karyotype; whereas chromosome abnormality is observed in most continuous cell lines (Hayflick, 1977). Sometimes continuous cell lines can give rise to tumors when injected into immunodeficient animals. They often lose the characteristic contact inhibition and grow to form multilayers. An exception to this generalization is the continuous 3T3 cell line (Todaro and Green, 1963). Green and Todaro proposed that those continuous cell lines are subsets of cells which survived crisis. Those cells were able to divide despite confluency. Thus, the establishment of cell lines involved the unwitting selection of those cells which outgrew other contact-inhibited cells. They carried out serial propagation of mouse cells through crisis without even allowing cells to remain in confluence for a long period, thus avoiding selecting those cells which have lost contact inhibition. The

3T3 cells thus established grew indefinitely in culture but were sensitive to contact inhibition of cell multiplication. This is the first fibroblast cell line that is not spontaneously transformed.

Cells detached from a surface after trypsinization are spherical. Cells that are suspended in a viscous fluid to retain their spherical shape do not divide. Farmer el al., 1978) showed that mRNA in such cells is stable but untranslatable. Protein and DNA syntheses are also suspended. Upon attachment to a compatible surface, cells begin to flatten, or spread out. This process of spreading is mediated by the copolymerization of cytoskeleton proteins, including microtubule, microfilaments and intermediate filaments. The recovery of protein synthesis follows reattachment to a surface as such, whereas mRNA, rRNA and DNA synthesis, as well as cell growth, require extensive cell spreading (Ben-Ze'ev el al., 1980). Interestingly, actin, a major component of the cytoskeleton, is translated in excess at the time of cell restructuring after cell attachment (Farmer et al., 1978). The close correlation between cell shape and cellular biosynthetic activity is clearly demonstrated in a study by Folkman and Moscoma (1978). As the cell shape changes from a sphere to a flattened sheet with an irregular perimeter, the height of the cells decreases. During this period the cell shape is affected by the growth cycle and cell density. Cells become more spherical, thus increasing in cell height, during mitosis. As they grow to confluence, each cell is laterally compressed by its neighbors and, thus, cell height also

increases. Folkman and Moscona used cell height as a measurement of cell shape and controlled the adhesiveness of the substrate by coating the plastic surface with varying concentrations of poly-(2-hydroxyethyl methacrylate) (poly(HEMA)). Cells exhibit their minumum height on the surface of maximum adhesiveness, in this case, the plastic surface without poly(HEMA) coating. The cell height decreases with increasing concentration of poly(HEMA) on the surface. The whole population plated on a surface could therefore be maintained at the same degree of spreading. Using H^3-thymidine incorporation as an index, they found cell shape to be tightly correlated to DNA synthesis. The flatter the cells, the more active was the synthesis of DNA. Even more striking was that, whenever the cell heights of two cultures were the same, normal diploid cells which were plated sparsely on a surface of controlled adhesiveness incorporated an amount of H^3-thymidine similar to that of confluent cells on the surface of maximum adhesiveness. Cell shape thus plays an important role in the growth control.

Cultured cells, even in spherical form in suspension, are not just droplets of cytoplasm surrounded by lipid-bilayers. Their shapes are determined by a subcellular protein lattice or cytoskeletal framework. The change in cell shape during growth and migration is achieved by the polymerization and depolymerization of the cytoskeletal proteins. These proteins can be visualized by electron or light microscopy. The relationship between cell shape and the cytoskeleton is very well illustrated in a study by Ben-Ze'ev et al. (1979). After cells

are lysed and soluble proteins and lipids are extracted, the cytoskeleton framework remains as a fibrous network which can be visualized by scanning electron microscopy. The fibrous, meshlike framework bears remarkable resemblences in cell morphology to intact cells. This is true for both flat fibroblasts and for spherical cells in suspension. Using radioiodination to label proteins external to cell surface before cell extraction, Ben-Ze'ev et al. showed that most proteins labelled externally remained on the surface sheet of the cytoskeletal frame work. Thus, the proteins on the external face of the lipid-bilayer are associated with cytoplasmic macromolecules.

In many cells, external to the membrane is a structure loosely called the extracellular matrix. The exact composition of the extracellular matrix is still to be elucidated but it has been found to be composed of glycosaminoglycan, proteoglycans (Culp et al., 1979) and glycoproteins, among them fibronectin (Chen et al., 1978). It was reported that a serum factor is required for cell attachment to substrate (Klebe, 1974). That serum factor is now believed to be fibronectin (for review, see Yamada and Olden, 1978). Using the antiserum specific for fibronectin, it can be shown that fibronectin exists as filamentous arrays over and under cells (Hynes et al., 1976). A large portion of these fibrils bridge between cells and lie between cells and substrate (Chen et al., 1976). After cells are detached from a substrate, fibronectin is left behind in the fibrils attached to the substrate (Hynes el al., 1976). Cells

adhering to the substrate rest upon the fibronectin-containing extracellular matrix.

For anchorage-dependent cells, a wettable surface is required for attachment to occur. Historically glass, which is wettable and contains negatively charged silicate or borate groups, was used as the culture surface. Presently, plastic surfaces which have been irradiated to give rise to negatively charged groups are most widely used (McKeehan et al., 1981). The most commonly used devices for anchorage-dependent cell growth are Petri dishes and roller bottles. These devices provide only a small growth surface area per unit vessel volume. They are not suitable for large scale cultivation of cells.

II. CELL CULTIVATION ON MICROCARRIERS

One of the most difficult problems hampering the commercialization of animal cell culture and its wide application for the production of biologicals is the inability to produce easily the large numbers of cells needed for product formation (Keary and Burton, 1979; van Wezel, 1981). The number of roller bottles required for large scale production of cells is staggering. Several attempts have been made to develop new systems for large scale operation. The major focuses have been on increasing the growth surface area per unit vessel volume and

on implementing convenient environmental control strategies. The devices developed toward these ends have been reviewed by Keary and Burton (1979) and Levine et al. (1979). One of the most promising advances in the field of cell culture technology has been the use of microcarriers, originally described by van Wezel (1967). van Wezel used the commercially available ion-exchange resin DEAE- Sepadex A50 as a support for anchorage-dependent cells. After pretreatment with nitrocellulose to diminish the apparent toxicity, the beads were capable of supporting the growth of monkey kidney primary cells. This development allowed the use of stirred tank reactors for anchorage-dependent cell cultures. However, the original microcarriers developed by van Wezel suffered from an apparent toxic effect. When the microcarrier concentrations were increased to a few grams per liter, cell growth usually deteriorated. Levine et al. (1979) synthesized DEAE-Sephadex beads with a lower ion exchange capacity. In the vicinity of 2.0 miliequivalents of DEAE per gram of dry Sephadex dextran, the microcarrier concentration can be increased several fold without the loss of cell viability or the appearance of any apparent toxicity. This in turn allowed cells to be grown in these microcarrier culture systems to a higher cell density. Microcarrier cultures have distinct advantages over conventional systems. These include a high ratio of growth surface area to reactor volume and a homogeneous environment, since one vessel replaces a large number of smaller ones. Another advantage is the potential for automatic environmental control, making large scale operation possible.

Since the introduction of controlled-charge microcarriers by Levine et al. (1979), there have been several studies on the application and the characterization of the microcarrier system. However, a great proportion of the publications were papers presented in scientific meetings and very little information regarding experimental conditions are available. Besides DEAE-Sephadex beads, many other types of microcarriers have been reported, including polyacrylamide beads (Mitchell and Wray, 1979; Reuveny et al. , 1983), polystyrene beads (Johansson and Nielsen, 1980; Kuo et al., 1981), hollow glass beads (Varani et al. , 1983), cylindrical cellulose beads (Reuveny et al. , 1982) and fluorocarbon droplets stablized with polylysine (Keese and Giaever, 1983). At present, DEAE-Sephadex-based microcarriers are those most widely used in the published literature. DEAE-polyacrylamide beads have been used in large-scale cultivation of cells (Feder and Tobert, 1983). Cylindrical cellulose derivatized with DEAE-charged groups supports the growth of chick embryonic fibroblasts in the form of cell-microcarrier aggregates, but does not support the growth of normal diploid cells (Reuveny et al. , 1982). Only preliminary results are available for fluorocarbon-, hollow-glass and polystyrene-based microcarriers. Both glass and polystyrene are used in conventional cultivation vessels. Polylysine can promote cell attachment and improve clonal growth of cells (McKeehan et al. , 1976). All of these types of beads may prove to be useful as microcarriers. Further studies are necessary to test their abilities to support the growth of a wide range of cell types and

to support product formation. One disadvantage of both polystyrene and glass beads is the light-reflecting properties of their surfaces, thus making microscopic examination of cell behavior difficult.

Microcarrier culture has been sucessfully applied to the cultivation of many cell types, including normal diploid fibroblasts (Levine et al. , 1979), epithelial cells such as Vero (Mered et al.,1980), and endothelial cells (Davies and Kerr, 1982). It has also been used in the production of vaccines for foot-and-mouth disease virus (Spier and Whiteside, 1976; Meignier, 1979), poliovirus (van Wezel et al. , 1979), and Herpes Simplex virus (Griffiths et al. , 1982) as well as in the production of β-interferon. At present, the chief area of application of cell culture is in the production of viral vaccines, e.g. killed poliomyelitis virus vaccine (van Wezel, 1981). Killed poliomyelitis vaccine is currently produced in primary monkey kidney cell culture at a cost which is greater than can be afforded by the nations which need it most (Hilleman, 1979). The cost of vaccine production can be greatly reduced by the use of a proper cell type in large scale microcarrier culture. For the production of proteins using genetically engineered cells, microcarrier culture is certainly one of the better systems for such large scale operation. However, some problems have to be overcome before large scale cultures can be implemented.

One of the most pressing problems has been the inability to inoculate a culture with a seed microcarrier culture, thus making

it necessary to inoculate with cells from roller bottles. The large number of roller bottles required for inoculation renders the inoculum acquisition a prohibitive task for large scale operations. In the study of tumor-associated oncornaviruses, Manousos et al. (1980) observed that a human rhabdomyosarcoma cell line grown on microcarriers could be detached after being exposed to Enzar-T, a commercially available protease mixture, or pronase, but not after exposure to trypsin. They also studied the growth of a human polyploid cell line chronically infected with cat virus in microcarrier culture. It was claimed that the addition of bare microcarriers to the culture allowed further cell proliferation on the newly added microcarriers. The kinetics of cell growth under such conditions were not, however, presented in that study; it is unclear how applicable those results are to other cell types. It is interesting to note that the medium used in that study was RPMI 1640, which is typically used for cell growth in suspension culture. The medium RPMI 1640 has a lower calcium concentration (0.43 mM) than DME medium (1.8 mM), which is widely used in the cultivation of anchorage-dependent cells. Calcium is implicated in cell-cell and cell-substrate binding, although the actual mechanism has not been elucidated (Morton, 1980). Depletion of calcium aids in cell dissociation with trypsin (Waymouth, 1974).

Using a medium which was low in calcium concentration (0.21 mM), Crespi and Thilly (1981) were able to allow cells to transfer to and grow on added bare microcarriers. This technique was applicable to two cell lines tested. One of them, Chinese

hamster ovary cells (CHO-K1), is capable of growing whether in suspension or attached; the other, monkey kidney cells (LLC-MK2), is a continuous, anchorage-dependent cell line. The most significant advantages of this technique are its simplicity and lack of requirement of trypsinization. However, calcium is a critical factor for cell growth. A reduction in calcium concentration can have a profound effect on the growth of many cell types. The concentration of calcium which gives rise to the maximum growth stimulatory effect is often in the vincinity of, or higher than 1 mM (Morten, 1980). In the study of clonal growth of both human diploid fibroblasts and chick embryo fibroblasts, McKeehan et al. (1981b) found that cell growth improves with increasing calcium concentration until the precipitation of calcium phosphate occurs at about 3 mM of calcium. For chick embryo fibroblasts, at a calcium concentration of 0.2 mM, the level used by Crespi and Thilly, the growth extent is only 30% of the maximum. Thus, the tolerances of the two cell types shown above, especially of the anchorage-dependent LLC-MK2 cells, and the continual propagation in low calcium medium are probably an exception rather than the rule. Another factor of consideration in using the low calcium technique is the cell distribution on microcarriers. Cell distribution on microcarriers can affect the observed growth rate. Those carried-over microcarriers having more cells are likely to reach confluence at an earlier stage than the newly added fresh beads. The actual rate may thus be lower than what is desired. The significance of the effect on the observed

growth rate is dependent on the rates of cell detachment, as well as reattachment, for both covered and newly added microcarriers. This technique will probably be useful for cell lines which grow to form multilayers and detach from the substrate readily when cell density is high. This would be for cells such as CHO and the tumor lines used in the previously mentioned study by Manousos et al. (1980).

In a recent publication, Feder and Tolbert (1983) reported a succesful detachment of human foreskin cells grown on polyacrylamide-based microcarriers. Using a settling tank, microcarriers were allowed to settle and to form cell-microcarrier aggregates. It was observed that cells could be detached from these aggregates after trypsinization. However, the experimental procedure and the results were not presented in their report. This technique may prove to be useful provided nutritional limitations during sedimentation will not be deteimental to the cells.

For many cell types, especially normal diploid cells, the requirement to prepare the inoculum from roller bottles has further complicated the task of initiating microcarrier cultures. Requirements of minimum inoculum concentration were observed for Vero (Mered et al., 1980) and FS-4 cells (Giard and Fleischaker, 1981) in order to achieve maximum growth rate and maximum growth extent. Decreasing the inoculum cell concentration resulted in a slower growth rate as well as a decreased growth extent. For FS-4 cells the large inoculum requirement resulted in a maximum achievable increase in cell number of only three- to four-fold in

a batch culture. Mered et al. also observed that the required inoculum concentration was affected by microcarrier concentration. In this study a cell density of 20,000 cells/cm^2 was reported optimal for Vero cells, but the reason for this was not addressed.

Oxygen, due to its low solubility, is often the limiting nutrient for the growth of microorganisms or animal cells in culture. Continuous sparging of air into liquid has been used in the cultivation of cells grown in suspension (Acton and Lynn, 1977), but this is not a common practice in cell culture. In most cases, oxygen is supplied by surface aeration. Telling and Radlett (1970) reported that oxygen supply was the limiting factor in the cultivation of baby hamster kidney cells grown in suspension. Even with pressurized pure oxygen in the headspace, the dissolved oxygen level in the bulk liquid was still below the optimal level for growth. Continuous mild sparging with air was found to be detrimental to these cells. Since the ratio of interfacial surface area to liquid volume decreases as reactor volume increases, the limitation of oxygen transfer will be more pronounced in large scale operation. In the study of chick embryo fibroblast cultivation on microcarriers, Sinskey et al. (1981) reported a decrease in maximum growth extent when the culture volume was increased from 100 ml to 1000 ml. The reduction in growth extent was attributed to oxygen transfer limitations in the one-liter culture. The use of gas-permeable silicone rubber tubing to increase the oxygen transfer area was proposed. Using silicone rubber tubing as an oxygenation system,

Fleischaker and Sinskey (1981) demonstrated that human foreskin cells, FS-4 could be cultivated in a seven-liter microcarrier culture without oxygen transfer limitation.

Another parameter sensitive to scale-up is the shear force exerted on the cells by fluid flow. In the scale-up of mixing processes, the maximum shear rate increases with impeller diameter at a constant impeller speed, while the average shear rate remains about the same at a given impeller speed regardless of impeller size (Oldshue, 1966). Using shear-sensitive protozoa as a model system, Midler and Finn (1966) found that extensive shear caused cell disruption. Their data showed a correlation between cell survival and impeller tip speed. Neither the Reynolds number nor the power input per unit volume seemed to be appropriate parameters. In microcarrier culture, the effect of shear force has not yet been extensively studied. Sinskey et al. (1981) studied the effect of shear force on the growth of chick embryo fibroblasts and subsequent use for virus production. It was found that the initial cell attachment was more shear sensitive than the subsequent growth. Both cell growth extent and virus productivity were impeded by extensive agitation. A parameter (integrated shear factor) based on the ratio of impeller tip speed to the tip-to-wall clearance was found to correlate with growth extent. At the agitation rate required to suspend microcarriers, the integrated factor is much lower than the value which is detrimental to cells. Furthermore, the power requirements necessary to suspend slurries decrease with

increasing scale (Zwietering, 1958); it was therefore concluded that excessive shear was unlikely to occur in large scale cultures.

Biotechnology is a rapidly progressing field. Its many impacts on our society cannot be overemphasized. Animal cell culture has long played a unique role in the biochemical industry. However, its potential role in biotechnology has only begun to be exploited. Whether animal cell culture will play a crucial role in the biotechnology industry is not only determined by the applied biologists who design the organisms but also by the engineers who have to overcome the many problems posed during cell culture.

MATERIALS AND METHODS

CELLS AND CELL CULTURE

Human foreskin fibroblasts, FS-4, were obtained from Dr. J. Vilcek of the New York University, School of Medicine, New York, New York and deposited at the Cell Culture Center, Massachusetts Institute of Technology. Cells were passaged and maintained in one liter roller bottles (490 cm^2) at 37 °C. One confluent roller bottle was used to inoculate four other roller bottles at each passage. The cells were frozen at passage number twelve in Dulbecco's Modified Eagle's (DME) Medium supplemented with 10% dimethylsulfoxide (DMSO) and 5% fetal calf serum (FCS). The frozen cells were thawed and propagated to passage number seventeen to nineteen prior to experimental use. The growth medium for newly passaged cells was DME supplemented with 5% FCS and 5% calf serum (CS). The compositin of DME is shown in Table I-1. FS-4 cells were maintained in a roller bottle for up to six weeks after having been inoculated from the previous passage. The medium was replenished weekly with DME supplemented with 2% FCS and 8% newborn calf serum (NCS). The continuous monkey kidney cell line, Vero, was obtained from Flow Laboratories (McLean, VA). The cultivation conditions were the same as those for FS-4 cells except that the DME medium was supplemented with 10% horse serum (HS) and each confluent roller bottle was used to

Table I-1. Composition of Media DME and F-12

Component	DME(mg/L)	F-12(mg/L)
$CaCl_2$	200.0	33.22
$CuSO_4 5H_2O$	---	0.00249
$Fe(NO_3)_3.9H_2O$	0.10	---
$FeSO_4.7H_2O$	---	0.834
KCl	400	224
$MgCl_2$	---	57.2
$MgSO_4$	97.7	---
NaCl	6400	7600
$NaHCO_3$	3700	1176
$NaH_2PO_4.H_2O$	125	---
Na_2HPO_4	---	142
D-Glucose	4500	1802
Hypoxanthine	---	4.10
Linoleic acid	---	0.084
Lipoic acid	---	0.21
Phenol red	15.0	1.20
Putrescine 2HCl	---	0.161
Sodium pyruvate	---	110
Thymidine	---	0.73
L-Alanine	---	8.90
L-Asparagine.H_2O	---	15.0
L-Arginine.HCl	84.0	211
L-Aspartic acid	---	13.3
L-Cystine	48.0	25.0
L-Glutamic acid	---	14.7
L-Glutamine	584	146
Glycine	30.0	7.51
L-Histidine HCl.H_2O	42.0	21.0
L-Isoleucine	105	3.94
L-Leucine	105	13.1
L-Lysine HCl	146	36.5
L-Methionine	30.0	4.48
L-Phenylalanine	66.0	4.96
L-Proline	---	34.5
L-Serine	42.0	10.5
L-Threonine	95.0	11.9
L-Tryptophane	16.0	2.04
L-Tyrosine	72.0	5.40
L-Valine	94.0	11.7
Biotin	---	0.0073
D-Ca pantothenate	4.00	0.480
Choline Chloride	4.00	13.960
Folic acid	4.00	1.30
i-Inositol	7.20	18.0
Nicotinamide	4.00	0.037
Pyridoxal HCl	4.00	0.062
Riboflavin	0.40	0.38
Thiamine HCl	4.00	0.340
Vitamin B_{12}	---	1.36

inoculate eight new roller bottles per passage. All media used were supplemented with penicillin G (100 units/ml) and streptomycin (100 μg/ml).

PREPARATION OF MICROCARRIERS

Microcarriers were prepared by the procedure of Levine et al. (1979) with some modifications. The desired bead size was obtained by sieving Sephadex G-50-80 or G-50-150. The microcarriers regularly used in this study were Sephadex beads having diameters from 53 to 75 μ. Larger Sephadex beads of diameters ranging from of 90 to 105 μ were also used in this work.

Diethylaminoethylchloride-hydroxychloride (DEAE.Cl-HCl) (Sigma Chemical Co., St. Louis, MO) was first recrystalized from methylene chloride before being used to prepare the DEAE-Sephadex beads. To prepare charged microcarriers, 40g of sieved dry Sephadex bceads were suspended in 480 ml of distilled water and placed in a two-liter round-bottom flask. Two hundred and forty ml of 2 M DEAE.HCl were added and the temperature of the reaction mixture was increased to 50°C by rotating in a water bath. After 30 minutes, the reaction was started by the addition of 240 ml of prewarmed 3 N NaOH. The reaction was carried out at 50°C by rotating the round bottom flask in a water bath. The reaction was allowed to proceed for one hour, and then quenched by the addition of one liter of distilled water. The beads were poured

into two 2-liter sintered glass funnels and were washed with 20 l of water followed by 12 l of 0.1 N HCl and 24 l of 0.0001 N HCl.

The charge density of microcarriers was quantified immediately after the washings. To measure the charge density, the microcarriers were washed with 10% (w/w) sodium sulfate (75 ml/g Sephadex G-50) and the effluent was collected. The effluent was then titrated with 1.0 N silver nitrate in the presence of potassium chromate indicator. Microcarriers prepared by this procedure typically have an ion exchange capacity of 1.8 to 2.1 meq/g dextran.

To prepare a microcarrier stock suspension, titrated microcarriers were washed with 25 volumes of distilled water followed by 40 volumes of calcium- and magnesium-free phosphate buffer saline(PBS). Washed beads were resuspended in PBS at 10 g/l, autoclaved, and maintained under sterile conditions until use.

MEASUREMENT OF MICROCARRIER DIAMETER

To measure the microcarrier diameter, the beads were suspended in PBS, placed onto glass slides and photographed at 100X magnification using an inverse microscope. A microscope ocular containing a micrometer (American Optics, NY) was used to measure the microcarrier diameter directly. The distribution of microcarrier diameters was obtained by either direct measurement

of the photographic prints or by an image processor (Magiscan) at the Center for Materials Science and Engineering at M.I.T. The number of microcarriers per milliliter was determined by direct counting using a grid glass plate. The median diamter of the microcarriers regularly used in this thesis varied from 170 to 185 μ in different separately prepared lots. The number of microcarriers per unit weight ranged from 4200 to 4600 per mg.

TITRATION OF MICROCARRIER CHARGE AS A FUNCTION OF PH

The procedure for titration of the charge of an ion exchanger at different pH values was described by Helfferich (1962). One gram of microcarriers in 100 ml of PBS was transferred to a sintered glass funnel and washed three times with 50 ml of a 1 N NaOH solution, followed by 50 ml of 0.15 N NaCl and transferred to a beaker. The microcarrier suspension was titrated with 0.1 N HCl with stirring. After each addition of 0.2 ml of the 0.1 N HCl solution, pH of the microcarrier suspension was allowed to stabilized befor it was measured with a pH meter.

CLONAL GROWTH ON PETRI DISHES

The procedure for clonal growth on Petri dishes was described by McKeehan et al. (1977). Cells were harvested from monolayer Petri dish cultures by trypsin treatment as follows: growth medium was first removed and cells were washed two times with a saline solution composed of 30 mM N-2-hydroxy-ethylpiperazine-N'-2-ethane sulfonic acid (HEPES)-NaOH buffer (pH 7.4), 4 mM glucose, 3 mM KCl, 130 mM NaCl, 1 mM $Na_2HPO_4.7H_2O$ and 0.0033 mM phenol red. Cells were then treated with a solution of 0.2% trypsin and 0.01% EDTA in PBS for 5 minutes and then dispersed. The dispersed cells were diluted in growth medium. The cell concentration was determined by counting with a hematocytometer and the suspension diluted to the desired concentration for inoculation. Each Petri dish (6 cm in diameter) was inoculated with 600 cells in 3 ml of medium. The dishes were incubated for 14 days in a carbon dioxide incubator saturated with water vapor. The gas phase carbon dioxide concentration used was 10 % for growth medium DME, 5 % for F-12 and 7.5 % for DME/F-12 (50:50) mixture. The composition of F-12 is shown in Table I-1. At the end of incubation, dishes were washed once with 3 ml PBS and stained with 0.1 % (w/v) crystal violet. Excess stain was washed away with PBS and the dishes were allowed to dry.

MICROCARRIER CULTURES

Unless otherwise noted, microcarrier cultures were seeded from roller bottle stock cultures. Two days prior to inoculation, cells from each roller bottle were trypsinized and inoculated into two seed roller bottles.

The 100 ml culture studies were carried out in spinner vessels (Wilbur Scientific Inc., Boston, Mass) containing a suspended magnetic impeller (0.8 cm x 4 cm) in a 250 ml flask (diameter 5.5 cm). The impeller was placed 1 cm above the bottom of the flask to avoid destruction of the microcarriers by shear. The spinner flasks were siliconized with 1% Prosil (VWR Sci. Co.) prior to use to prevent microcarriers sticking to vessel walls.

Unless otherwise specified, the medium used for microcarrier culture was DME. For FS-4 cells, the medium was supplemented with 5% FCS and for Vero cells, with 10% horse serum. A microcarrier concentration of 5 g/l was normally used. To prepare a spinner culture, 0.5 g of microcarriers in PBS were allowed to settle and were washed twice with 30 ml of medium without serum. The microcarriers were then reconstituted to 80 ml with serum-supplemented medium and transferred to the spinner. Spinners were placed in a humidified carbon dioxide incubator to allow the temperature to equilibrate before cells were inoculated.

The inoculum was obtained by trypsinizing cells from seed roller bottles using 0.2% trypsin in PBS with 0.02% ethylenediamine-

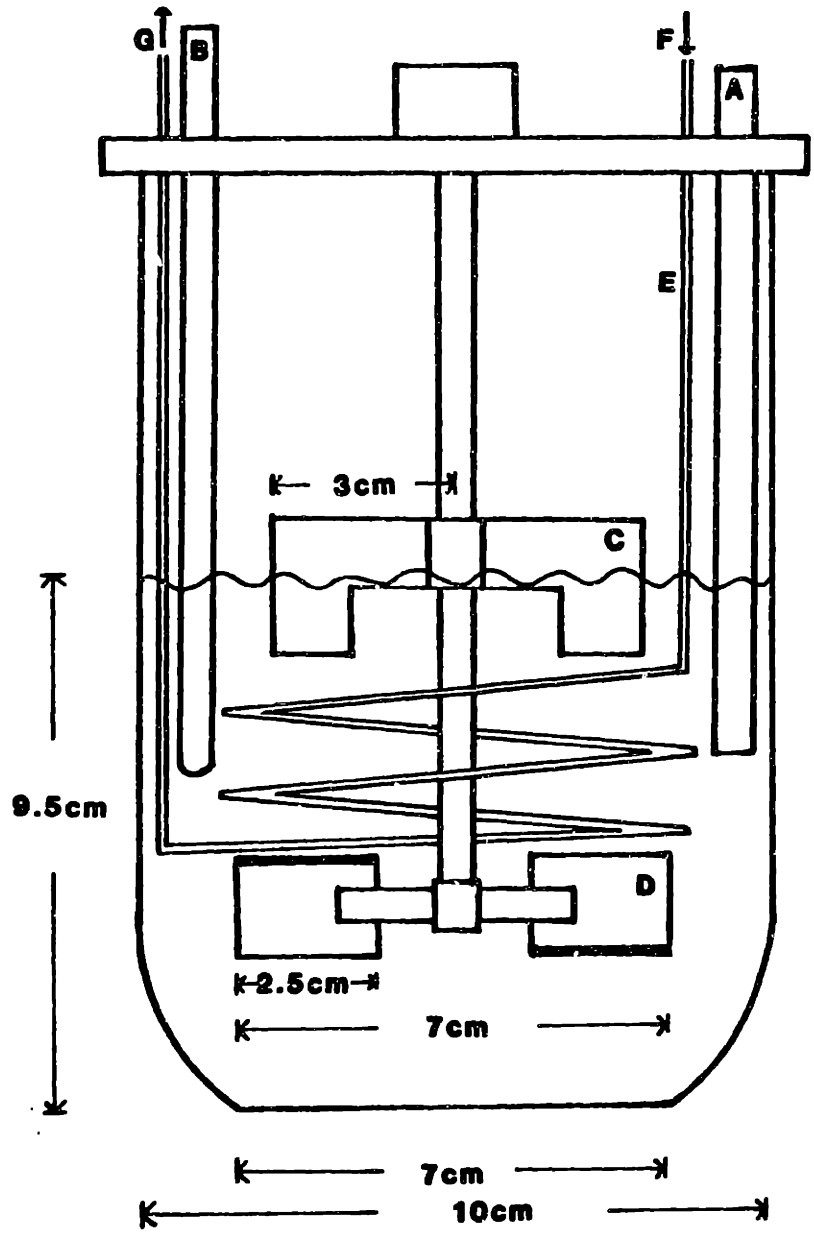
tetraacetic acid (EDTA). Cells were pelleted by centrifugation at 800xg for ten minutes. The pelleted cells were resuspended in the culture medium and the viable cell concentration was determined by the dye exclusion method using a hematocytometer. The dye employed was 0.1% trypan blue in PBS. The volume of the inoculum was then increased to 20ml with the prewarmed medium and inoculated into the microcarrier suspension. An agitation speed of 65 to 75 rpm was used.

For the cultivation of FS-4 cells, 50% of the culture medium was removed and replenished with fresh medium on the third day. For those experiments which lasted longer than six days, additional 50% medium changes were performed on the sixth and eighth days. More frequent medium change was necessary for Vero cells. The first 50% medium change was performed about sixty hours after inoculation. After the fourth day, the medium was changed every day.

For studies involving larger diameter microcarriers, the impellers of the spinners were modified. Two 45 degree pitched blades (2.2 x 2.2 cm) were installed onto the paddle to allow a lower agitation rate (45 rpm) to be used.

The 500 ml cultures were carried out in one liter vessels. A schematic diagram of the 1L vessel used in this study is shown in Figure I-1. Eighty cm of silicone rubber tubing (0.058 in. i.d., 0.077 in. o.d., silastic tubing, Dow Corning) were placed in the vessel. A surface aerator was used for the cultivation of Vero cells to improve oxygen transfer. For FS-4 cells, the dissolved oxygen level could be maintained above 40% of the

Figure I-1. One Liter Vessel with the Surface Aerator for Microcarrier Culture. The dimensions are described in the Figure. (A): pH electrode, (B): dissolved oxygen electrode, (C): surface aerator, (D): impeller with two 45 degree pitched blades, (E): silicone rubber tubing, (F): air inlet, (G): air outlet.


G
B
F
A
E
3cm
C
9.5cm
D
2.5cm
7cm
7cm
10cm

saturation level with air without the use of silicone rubber tubing. In the DME medium, the pH of the culture ranged from 7.15 to 7.35 for FS-4 cells and from 7.00 to 7.35 for Vero cells. However, when the DME/F12 mixture was used for the FS-4 cells, pH decreased significantly in the absence of pH control. For those experiments, pH was controlled by activating a controller allowing air to pass through silicone rubber tubing, reducing the dissolved CO_2 concentration. The air flow rate through the silicone rubber tubing was about 100 ml/min.

Cell growth was monitored according to the method reported by van Wezel (1967) and based on the original technique of Sanford et al. (1949). A 2 ml sample was withdrawn from a well-mixed spinner vessel and then centrifuged at 500xg for three minutes. The supernatant was removed and the pelleted microcarriers were resuspended in 2 ml of 0.1% (w/w) crystal violet in 0.1 M citric acid. After incubation for one hour at 37 °C, the suspension was sheared using a Pasteur pipette to detach the nuclei from the microcarriers. The stained nuclei were then counted in a hematocytometer.

CELL ATTACHMENT TO MICROCARRIERS

The cell attachment experiment was performed in 250 ml spinner flasks. For each spinner flask, 0.5 gram of microcarriers was added to 60 ml of medium. The microcarrier

suspension in the spinner was placed in an incubator to allow the pH and temperature to reach 7.3 to 7.4 and 37 °C respectively. A cell suspension in 40 ml medium was added to the microcarrier suspension and the mixture was agitated at 65 rpm. To measure the kinetics of cell attachment, the microcarriers were allowed to settle and the the supernatant was carefully withdrawn for cell enumeration with a Coulter counter.

MEASUREMENT OF OXYGEN CONSUMPTION RATE

A 50 ml flask containing a suspended magnetic stirring bar and a dissolved oxygen probe was used to measure the oxygen consumption rate of cells. When the flask was filled with liquid, no head space or air bubbles remained inside the vessel; thus no transfer of oxygen into the liquid was possible during the measurement. The change in the dissolved oxygen concentration was therefore due to consumption by cells. To measure the oxygen consumption rate, cells on microcarriers were withdrawn from the culture vessel and the dissolved oxygen level of this suspension was raised to nearly saturation with air by shaking gently and then placed into the 50 ml flask described above. The flask was then filled with medium to eliminate the remaining head space and then agitated at 65 rpm. The decrease in the dissolved oxygen level was recorded. The oxygen consumption

rate was calculated as the rate of decrease in dissolved oxygen divided by the cell concentration in the flask.

MEASUREMENT OF OXYGEN TRANSFER COEFFICIENT

The oxygen transfer coefficient for the one-liter vessel was measured both with and without the surface aerator. The measurement was carried out with 500 ml of PBS at 37 ^{o}C. The dissolved oxygen in the PBS was first depleted by sparging with nitrogen gas. Air was then introduced to the head space and the change in dissolved oxygen concentration in the PBS recorded. The volumetric oxygen transfer coefficient (K_La) was determined by measuring the slope of the plot of $\ln(C^*-C_L)$ as a function of time. In the above, a is the interfacial area per unit volume (cm^{-1}) of liquid; K_L is the mass transfer coefficient (cm/sec); C^* is the dissolved oxygen concentration at equilibrium with air(mmoles/ml); and C_L is the dissolved oxygen concentration in the liquid (mmoles/ml).

MEASUREMENT OF TRYPSIN ACTIVITY

The proteolytic activity of trypsin was measured with a chromogenic substrate, azocollagen (azocoll). The azocoll (Sigma

Chemical Co.) was prepared at 10 mg/ml in 30 mM buffer of different pH's. The trypsin solutions at various pH's were prepared by diluting ten-fold concentrated solution in 30 mM HEPES buffer. The trypsin concentration was 0.2%. To perform the assay, 4ml of the trypsin solution were added into each test tube containing 4 ml of substrate solution at the same pH. The mixture was incubated at 37 ^{o}C for 15 min. One ml of sample was withdrawn periodically. After filtering through filter paper, the color intensity of the filtrate was read on a spectrophtometer (520 nm).

DETACHMENT OF CELLS FROM MICROCARRIERS

To trypsinize cells from microcarriers, microcarriers were first withdrawn from a spinner flask and placed in a 250-ml plastic centrifuge tube. After the supernatant medium was removed, cells and microcarriers were washed extensively with 30 volumes (ml/ml beads) of PBS. The trypsin solution was prepared by diluting a ten-fold concentrated stock solution in a saline solution composed of 30 mM HEPES buffer, 4 mM glucose, 3 mM KCl, 130mM NaCl, 1mM Na_2HPO_4 and 0.0033mM phenol red (McKeehan et al., 1977). The diluted trypsin solution contained 0.2% trypsin and 0.01% EDTA. The trypsin solution could also be prepared in PBS with pH adjustment. The pH was adjusted with 2 N NaOH to 8.9 to

9.0. Later in the study, solutions of pH 8.2 to 8.4 were also used to detach cells successfully.

The trypsinization was carried out in a sintered glass funnel (50 ml) or in a chromatographic column (2.5 x 10 cm) (Biorad Co., NY) to facilitate the removal of excess trypsin solution. To each 10 ml of washed microcarrier pellet, 50 ml of trypsin solution were added. The microcarrier suspension was then transferred to the sintered glass funnel or the chromatographic column and the microcarriers were allowed to settle and the excess trypsin to flow through the bed formed by microcarriers. A portion of the microcarriers was withdrawn periodically for microscopic examination. After cell shape became more spherical, the residual trypsin was removed by suction from the top of the microcarrier bed. When a low multiplication ratio (lower than four) was used, the cells and microcarriers were washed with one volume of DME supplemented with 5% FCS (or 10% HS for Vero cells) to remove residual trypsin from the microcarriers. When a high multiplication ratio was used, the amount of trypsin carried over to the next cultivation step was small, and the last washing with serum-containing medium was therefore omitted. The duration of trypsinization varied from 3 to 10 min. depending on the pH used. In all cases studied, the morphological change was used as the criterion rather than the duration of trypsinization.

After trypsinization, microcarriers were suspended in prewarmed medium. Cells thus treated could be detached by repeated pipetting if the volume to be handled was small. For

larger volumes, the microcarrier suspension was poured through a 30 cm high, 1.5 cm diameter column packed with glass beads having a diameter of about 3 mm. The bed height of glass beads was 20 cm. Typically more than 90 % of the cells on the beads were released under such conditions. If a significant portion of cells remained attached, the microcarrier suspension was repeatedly passed through the column. The cell suspension along with the used microcarriers was then inoculated into a new culture vessel. The inoculation procedure was the same as described in the microcarrier culture section of Materials and Methods. Under the conditions used, cells reattached to microcarriers within half an hour after inoculation.

VESICULAR STOMATITIS VIRUS (VSV) PRODUCTION

The production of VSV as described by Giard et al. (1977) was achieved using a 500 ml culture of Vero cells in a one liter vessel. The multiplicity , defined as the ratio of viral plaque forming units (PFU) to cell number, used for the infection was 0.1. Before the addition of VSV, microcarriers were allowed to settle and 250 ml of the medium were removed to reduce the culture volume. After the addition of VSV, the pH of the microcarrier suspension was decreased to 6.8 by the addition of CO_2. In the following one hour period, pH was maintained between 6.5 and 6.8. and the culture was agitated only occasionally,

about one minute in every ten to allow viruses to adsorb onto the cells. Subsequently, 250 ml of medium were added to bring the culture volume back to 500 ml. The pH was readjusted to 7.3 and the agitation resumed. A 5 ml sample was withdrawn periodically for the titration of the virus concentration. The samples were centrifuged at 1,000xg at 4 °C for 10 minutes to remove cell debris. The supernatant was frozen at -20°C until plaque assays were performed.

VIRUS PLAQUE ASSAY

The VSV concentration was titrated by the plaque assay, using secondary chick embryo fibroblasts. Ten-day-old chick embryos were used to establish the primary culture. Two days prior to the plaque assay, confluent primary chick embryo fibroblasts were trypsinized and inoculated to 6 cm diameter Petri dishes to start secondary cultures. The medium used was DME supplemented with 1% chick serum, 2% tryptose phosphate broth and 1% calf serum.

To perform the plaque assay, the medium of the confluent secondary cultures on Petri dishes was first removed. Serial ten-fold dilutions were made with each sample and 0.2 ml was inoculated onto each dish. Each sample was assayed in duplicate. The virus was allowed a one-hour adsorption period at 37°C, in a humidified 10% CO_2 atmosphere. After adsorption, 2 ml of 1%

Noble agar at 40 °C were mixed with 2 ml of two-fold concentrated DME medium supplemented with 10% FCS at 37 °C and overlayed onto each dish and the dishes were then incubated at 37 °C for two days. Plaques were scored after the dishes were stained with 1:2,500 dilution of neutral red solution.

INTERFERON PRODUCTION

The procedure for interferon production has been described (Giard et al., 1981) based on the original procedure of Havell and Vilcek (1972). This procedure involves three stages: priming, induction and production.

The priming was carried out 37 °C. FS-4 cells on microcarriers were first washed two times with DME without serum, and then resuspended in DME supplemented with 1% FCS. The cell concentration was between 1.0 and 1.2 x 10^6 cells/ml. After the addition of fibroblast interferon at a concentration of 50 units/ml, the priming was allowed to proceed for sixteen hours.

To start induction, cells were first washed twice with DME without serum. The induction was carried out in DME without serum and at 34°C. The inducer, poly I.poly C (PL Biochem. Co., Milwakee, WI), was added at 50 μg/ml together with 10 μg/ml of cycloheximide (Sigma Chem. Co.). After 4 hr of incubation at 34°C, actinomycin D (Sigma Chem. Co.) was added to give a concentration of 1 μg/ml. After 2 hr of further incubation, the

medium was removed and the cells washed twice in DME and subsequently resuspended in the production medium.

The production medium was DME supplemented with 0.5 % plasmanate. The initial temperature was 37°C. After one hour, the production temperature was shifted to 30°C. The supernatant was collected and fresh medium added after 24 hr. At 48 hr, the medium was collected again and the microcarriers were discarded. The culture fluids were centifuged at 1000xg for 10 min and the supernatant frozen at -70°C until assayed for β-interferon.

INTERFERON ASSAY

The assay of the inhibition of virus-induced cytopathic effect (CPE) described by Havell and Vilcek (1972) was used to measure the interferon produced by the FS-4 cells. The samples were assayed in duplicate using 96-well microplates. One-tenth ml of medium (DME supplemented with 2% FCS) was added to each well. Serial two-fold dilutions of prediluted samples were performed in each row of twelve wells. To each well 5 x 10^4 FS-4 cells in 100μl of growth medium were added. The plates were then incubated for 24 hr at 37°C before cells were challenged with 10,000 PFU of VSV (Indiana strain) per well. The plates were further incubated for 48 to 72 hr until the control wells, to which no interferon was added, showed total cell destruction. The plates were scored microscopically and the highest dilution

of the sample showing 50% destruction of cells was considered the endpoint. An internal standard calibrated against the International standards G023-901-527 and G023-902-527 (obtained from National Institutes of Health, Bethesda, MD) was included with each assay.

RESULTS AND DISCUSSION

CHAPTER 1. MODELLING OF CELL GROWTH AND IMPROVEMENT OF MULTIPLICATION RATIO

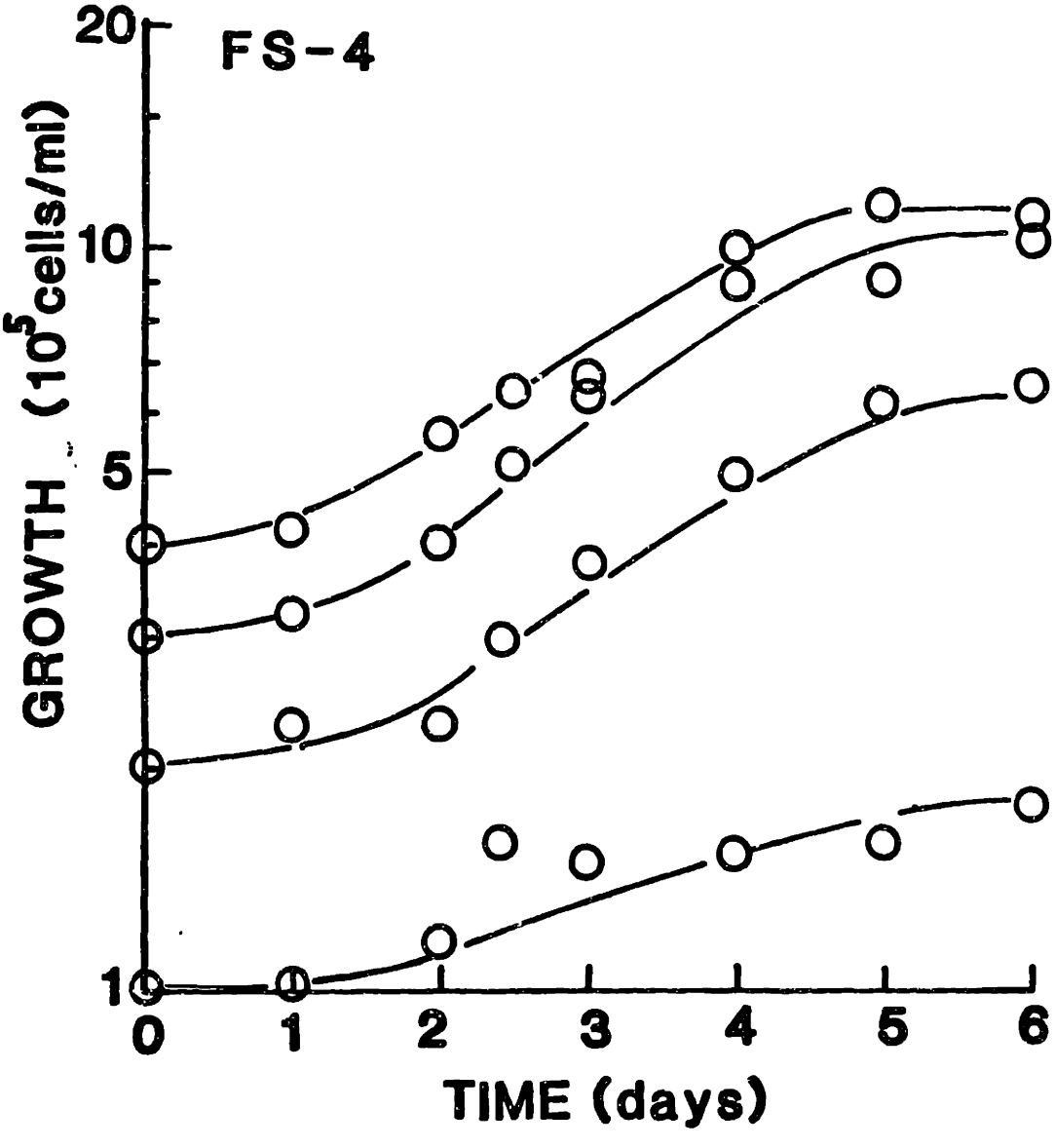
1.1 A MECHANISTIC ANALYSIS OF THE INOCULUM REQUIREMENT

1.1.1 EFFECT OF INOCULUM CELL CONCENTRATION

To study the effect of inoculum, FS-4 cells were inoculated at different concentrations into cultures containing 5 g/l of microcarriers. The results are shown in Figure 1-1. At this microcarrier concentration, the maximum extent of growth attainable was 1.2 x 10^6 cells/ml. This was achieved when an inoculum concentration higher than 3 x 10^5 cells/ml was used. Decreasing the inoculum cell concentration to 2 x 10^5 cells/ml resulted in a decrease in the final growth extent. Further reduction to 1 x 10^5 cells/ml resulted in drastic reduction of both growth rate and extent of growth. Thus only three- to four-fold increases in cell number could be achieved in batch culture of FS-4 cells on microcarriers when a sufficiently high inoculum was used to achieve maximum growth extent. This is

Figure 1-1. Effect of Inoculum Concentration on the Growth of FS-4 Cells on Microcarriers.

Microcarrier concentration was 5 g/l. Cultures were inoculated at the cell concentrations shown in the figure. The medium was DME supplemented with 5% FCS.

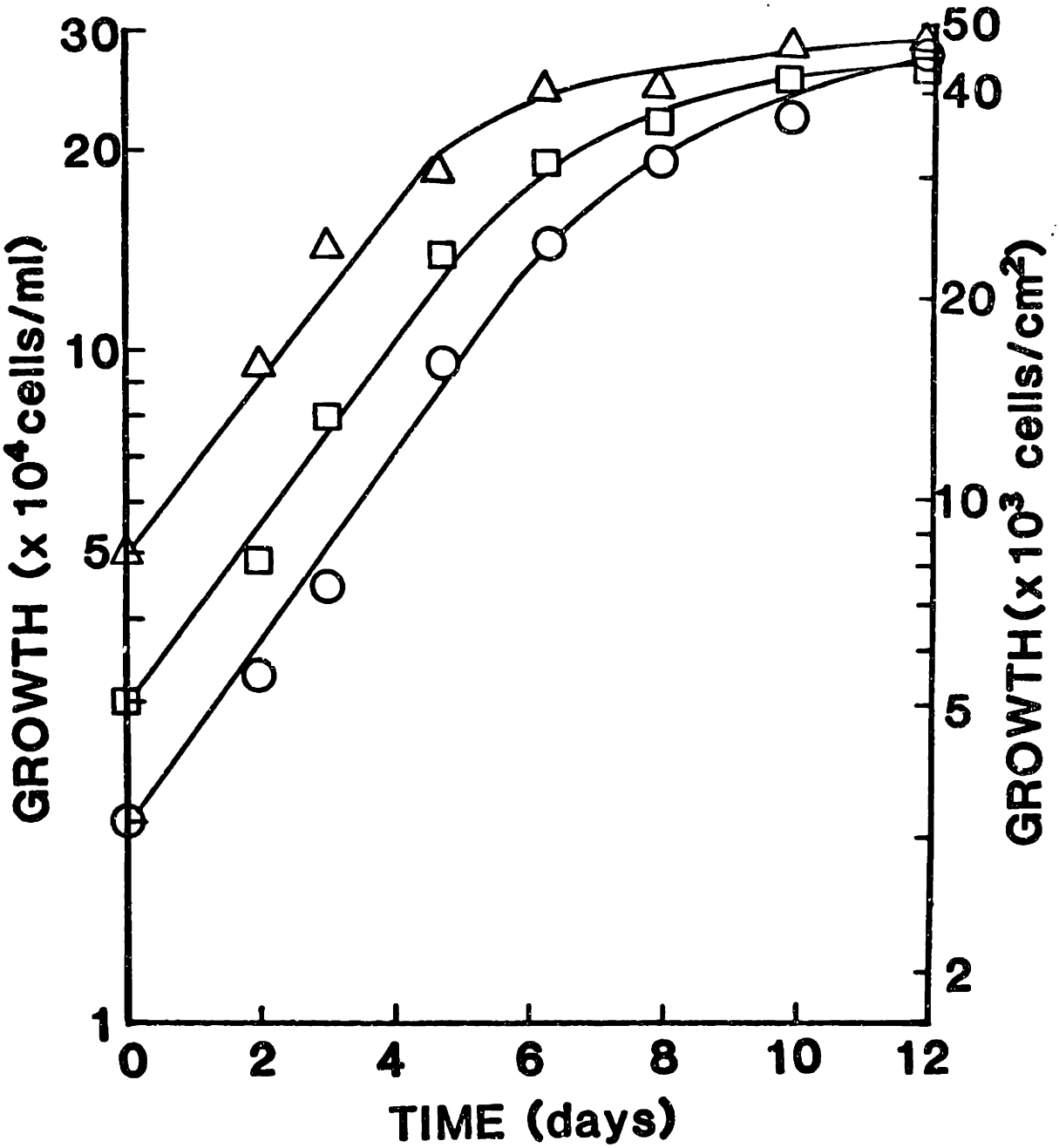

FS-4
GROWTH (10^5cells/ml)
20
10
5
1
0
1
2
3
4
5
6
TIME (days)

consistent with the findings reported by Giard and Fleischaker (1980).

Shown in Figure 1-2 are the growth kinetics of FS-4 cells on Petri dishes at different inoculum concentrations. In contrast with growth on microcarriers, the growth of FS-4 cells on Petri dishes was less sensitive to reduction of the inoculum concentration. Although a multiplication ratio of four was routinely used for cell maintenance, a fifteen-fold increase in cell number could be achieved in a batch culture on conventional plastic surfaces.

It was reported more than two decades ago that the nutritional requirements for growth at high cell concentration might be different from those at low cell concentration (Eagle and Piez, 1962). It was noted that at high cell concentrations, the growth of cells did not require the addition of serine, asparagine, cystine, glutamine, homocystine, inositol and pyruvate, while at low cell concentrations, these nutrients became essential. Factors produced by cells in mass cell culture which are essential for the growth of the same cell type at low inoculum concentration or for the growth of a second cell type were called conditioning factors. The medium in which cell growth had occurred was called conditioned medium. In another earlier finding, Puck and Marcus (1955) showed that a layer (feeder layer) of irradiated, nondividing but still metabolically-active HeLa cells could provide the conditioning factor(s) required for the formation of colonies by the HeLa cells even though inoculated at a very low cell concentration.

Figure 1-2. Growth of FS-4 Cells on Petri Dishes at Different Inoculum Concentrations.

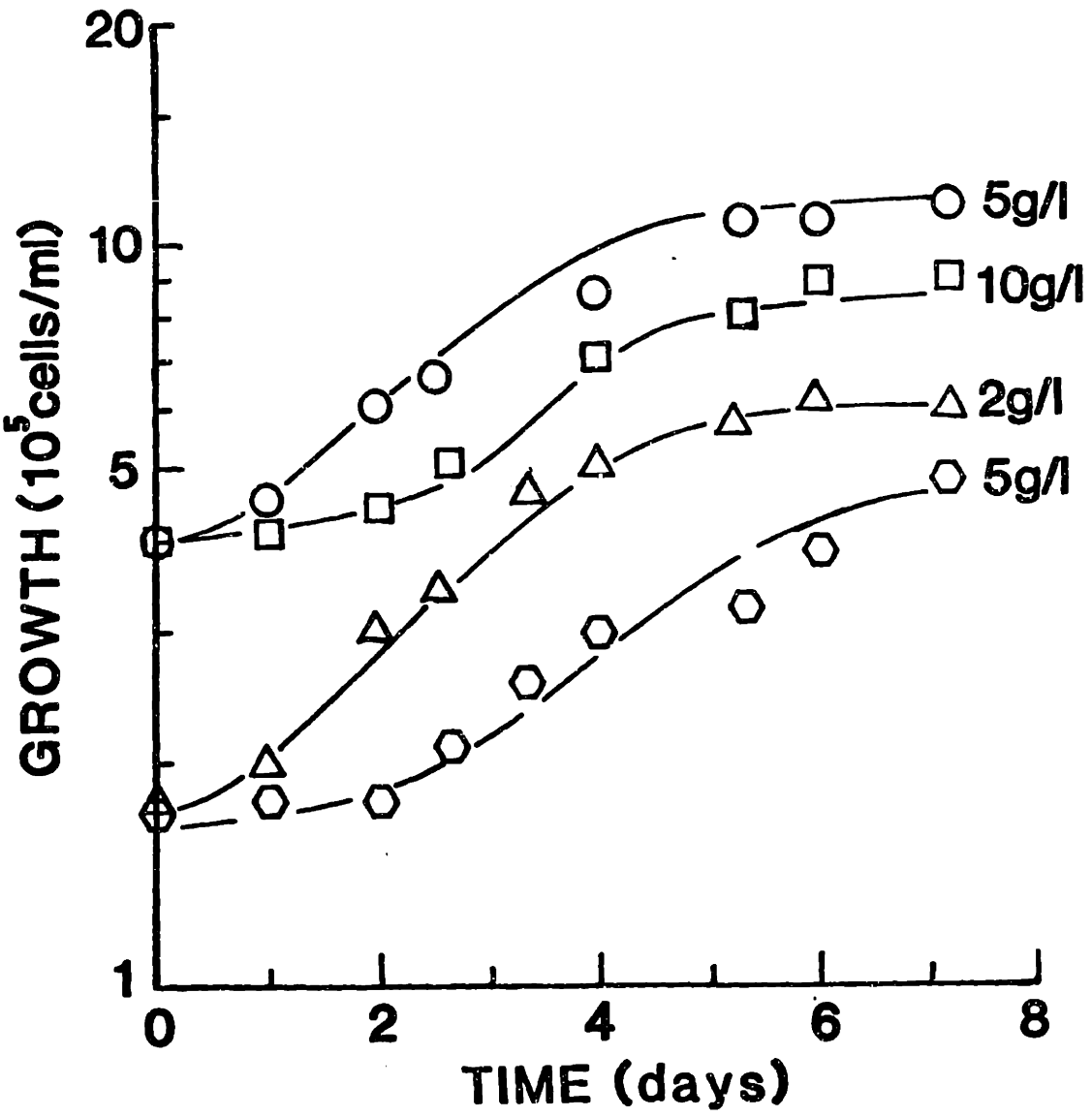

30
20
10
5
1
GROWTH (x 10⁴cells/ml)
50
40
20
10
5
2
GROWTH (x 10³ cells/cm²)
0
2
4
6
8
10
12
TIME (days)

The effect of inoculum concentration was due at least in part to the diffusible conditioning factors. This requirement of the conditioning factors could also be met by the addition of proper nutrients. In these studies, the ratio of the growth surface area to the liquid volume was relatively constant; varying cell concentration on a per unit volume basis inevitably varied the cell density on a per unit surface area basis. Because the conditioning factors were diffusible molecules, the inoculum requirement for cell growth on conventional surfaces was considered to be dependent on a per unit volume basis.

In the results shown in Figure 1-1, the same microcarrier concentration was used for the different inoculum concentrations. This population dependence could be solely a cell concentration dependency, or it could be dependent on the microcarrier concentration.

To determine whether the inoculum requirement in microcarrier culture was genuinely dependent only on cell concentration per unit medium volume or on the cell number per unit amount of microcarriers instead, both the microcarrier concentration and the inoculum concentration were varied. The results are shown in Figure 1-3. An inoculum concentration of 4 x 10^5 cells/ml was used to initiate cultures containing 5 g/l and 10 g/l of microcarriers. Consistent with the results shown in Figure 1-1, at 5 g/l of microcarriers, cells grew to confluence. However, at 10 g/l microcarrier concentration, although more surface area was available for cell growth, the extent of growth was not greater than that at 5 g/l. At the inoculum

Figure 1-3. Effect of Microcarrier and Inoculum Cell Concentrations. Inoculation cell concentrations are shown on the figure. Microcarrier concentrations were: (Δ), 2 g/l; (o,O), 5 g/l; (□), 10 g/l.

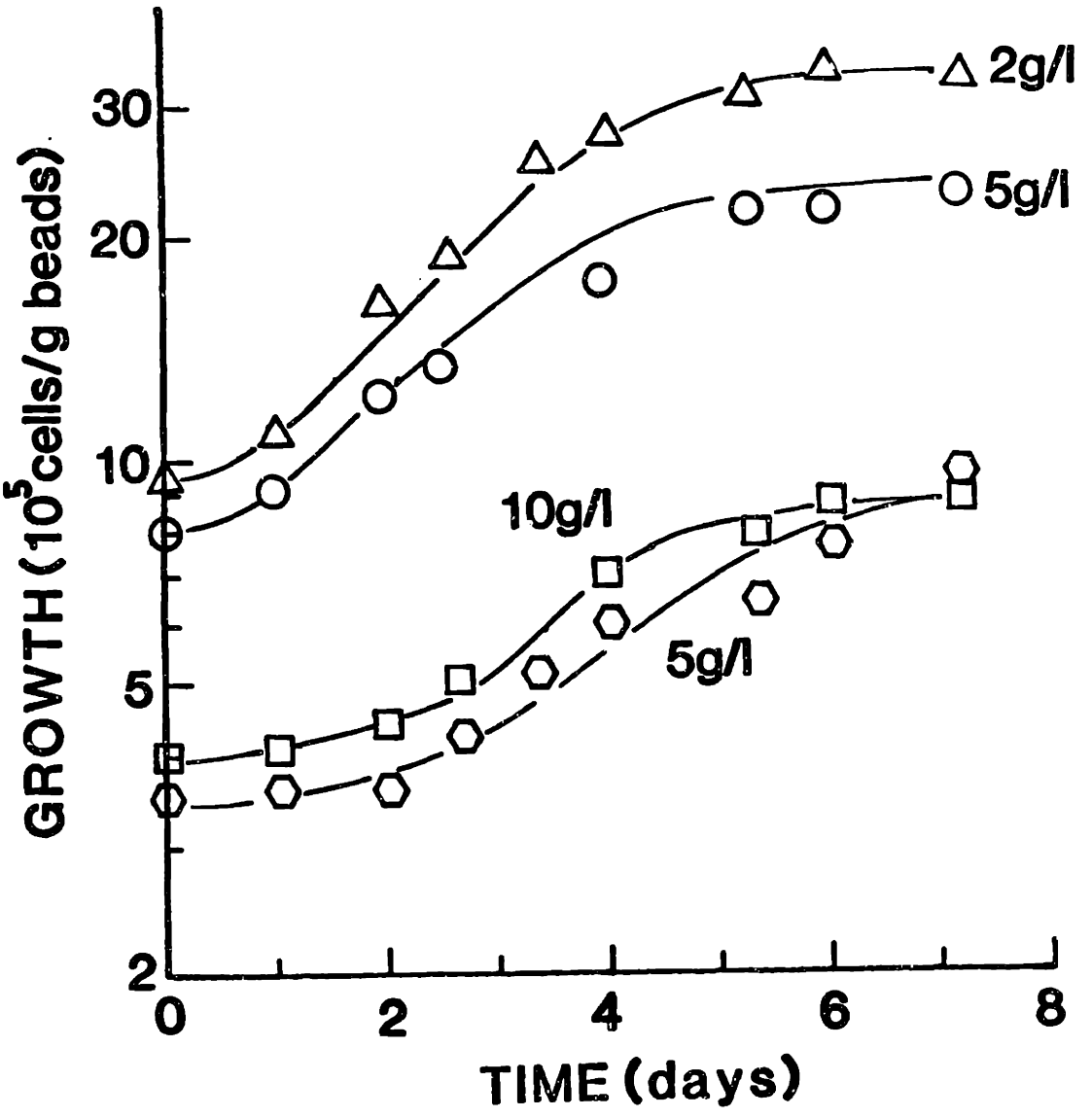

20
10
5
1
GROWTH (10^5cells/ml)
0
2
4
6
8
TIME (days)
5g/l
10g/l
2g/l
5g/l

concentration of 2 x 10^5 cells/ml, confluent growth could not be achieved at 5 g/l. At a reduced microcarrier concentration of 2 g/l, however, cells grew normally to confluency.

Since confluent growth could be attained with either normal or reduced inoculum based on per unit medium volume, the inoculum requirement was concluded not to be dependent on the absolute cell concentration. Furthermore, since confluence could be achieved with a concurrent reduction in both cell inoculum and microcarrier concentrations, it was postulated that the dependence on inoculum concentration might be related to the microcarrier concentration. In order to test this postulation, the results were analyzed further in another fashion.

The cell growth at different microcarrier concentration from Figure 1-3 was expressed as the number of cells per gram of microcarriers. The cell growth normalized to microcarrier concentration was replotted as shown in Figure 1-4. The normalized inoculum cell density is related to subsequent growth. The cells inoculated at a higher cell number per gram microcarriers grew to near confluence (2.4 x 10^8 cells/g microcarriers), while those inoculated at a lower cell number per gram microcarriers never reached a comparable cell density. These results showed that the required inoculum concentration was dependent on microcarrier concentration. For FS-4 cells, an inoculum concentration greater than 6 x 10^7 cells/g microcarriers was necessary.

Figure 1-4. Effect of Inoculation Cell Density on Subsequent Growth. Symbols are the same as those in Figure 1-3.


GROWTH (10^5cells/g beads)
30
20
10
5
2
0
2
4
6
8
TIME (days)
2g/l
5g/l
10g/l
5g/l

1.1.2 EFFECT OF MICROCARRIER CONCENTRATION ON CELL GROWTH

The ion exchange-property of microcarriers has often been speculated to deplete or to reduce the concentrations of some essential nutrients or conditioning factors (Horng and McLimans, 1975) in cell culture medium. It is thus possible that a minimum cell number per unit weight of microcarriers is needed to provide sufficient conditioning factors to avoid their being completely adsorbed or depleted by the microcarriers. In this case, the inoculum requirement would be affected by a diffusible molecule(s); high microcarrier concentration per se could affect the growth. Since the effect is exerted by a diffusible molecule(s), physical contact between the effectors (microcarriers) and the affected (cells) does not determine the growth behavior. The average number of cells per gram microcarriers would thus be critical for cell growth.

On the other hand, the requirement for a minimum inoculum concentration for a given microcarrier concentration could be caused instead by the microenvironment of the microcarriers. A unique property of microcarriers is the discreteness of the substrate on which cells grow. Except for sharing diffusible molecules in the bulk medium, each microcarrier is a separate domain and each has its own microenvironment. Cell attachment to the microcarriers after inoculation is a probabilistic phenomenon. The number of cells per microcarrier varies and most probably follows some distribution function. The

microenvironment could be dependent upon the number of cells attached to a microcarrier. A change in the number of cells per gram of microcarriers would change the cell distribution on the microcarriers, thus changing the frequency distribution of the microenvironments.

Therefore, the requirement of a minimum ratio of inoculum concentration to microcarrier concentration could be due to a dependence on a critical number of cells per gram of microcarriers. Alternatively, it could reflect a dependence on the cell distribution on microcarriers associated with the observed average cell number per microcarrier.

The distribution function of cells on microcarriers does not change after attachment. However, the average number of cells per gram of microcarriers can be changed, without affecting cell disribution on microcarriers already present, by adding new microcarriers with no cells attached. The effect of the average cell number per gram of microcarriers and the cell distribution on microcarriers could thus be differentiated through the addition of new microcarriers to a culture after cell attachment.

Such an experiment was performed with two initial microcarrier concentrations. As controls, cells were inoculated at a concentration of 4×10^5 cells/ml into vessels containing 5 g/l of microcarriers, and at 1.7×10^5 cells/ml into spinners containing 2 g/l of microcarriers. Separate vessels were prepared under identical conditios, and after cell attachment and microscopically observable cell spreading had occurred, new

microcarriers were added 48 hours after inoculation. In these two cases, the total microcarrier concentrations were 10 g/l and 5 g/l respectively. Growth was followed and compared to that of the controls to which no new microcarriers were added. The results in Figure 1-5 showed that the addition of new microcarriers to cultures after cells had attached did not affect either the cell growth rate or the growth extent. A similar experiment was performed in which cells were inoculated at 3.5 x 10^5 cells/ml to a culture containing 5 g/l of microcarriers. Subsequently, 10 g/l of microcarriers were added at 16 hr to bring the total microcarrier concentration to 15 g/l. The results are shown in Figure 1-6. Again, the addition of new microcarriers at the concentration used did not affect cell growth. A mixture of bare carriers and confluent carriers was observed at the end of the cultivation period. This behavior indicates that once the cells have attached to a microcarrier, they do not detach and reattach to other carriers to any appreciable extent.

The effect of inoculum concentration on cell growth on microcarriers has also been reported for Vero cells by Mered et al. (1980). Using the same microcarrier concentration while varying inoculum concentrations, they found that an optimum inoculum cell concentraion was necessary to achieve maximum growth extent. They expressed the inoculum requirement in cell density (20000 cell/cm^2), but did not speculate on the cause of this phenomenon. Using this optimum inoculum cell density, they compared cell growth at microcarrier concentrations of 1.0, 2.5,

Figure 1-5. Effect of Addition of New Beads to Microcarrier Cultures. Microcarrier concentrations were (o): 5 g/l; (□): 5g/l, at 48 hr new microcarriers were added to 10 g/l; (Δ): 2g/l; (⬡): 2 g/l, at 48 hr new microcarriers were added to 5 g/l.

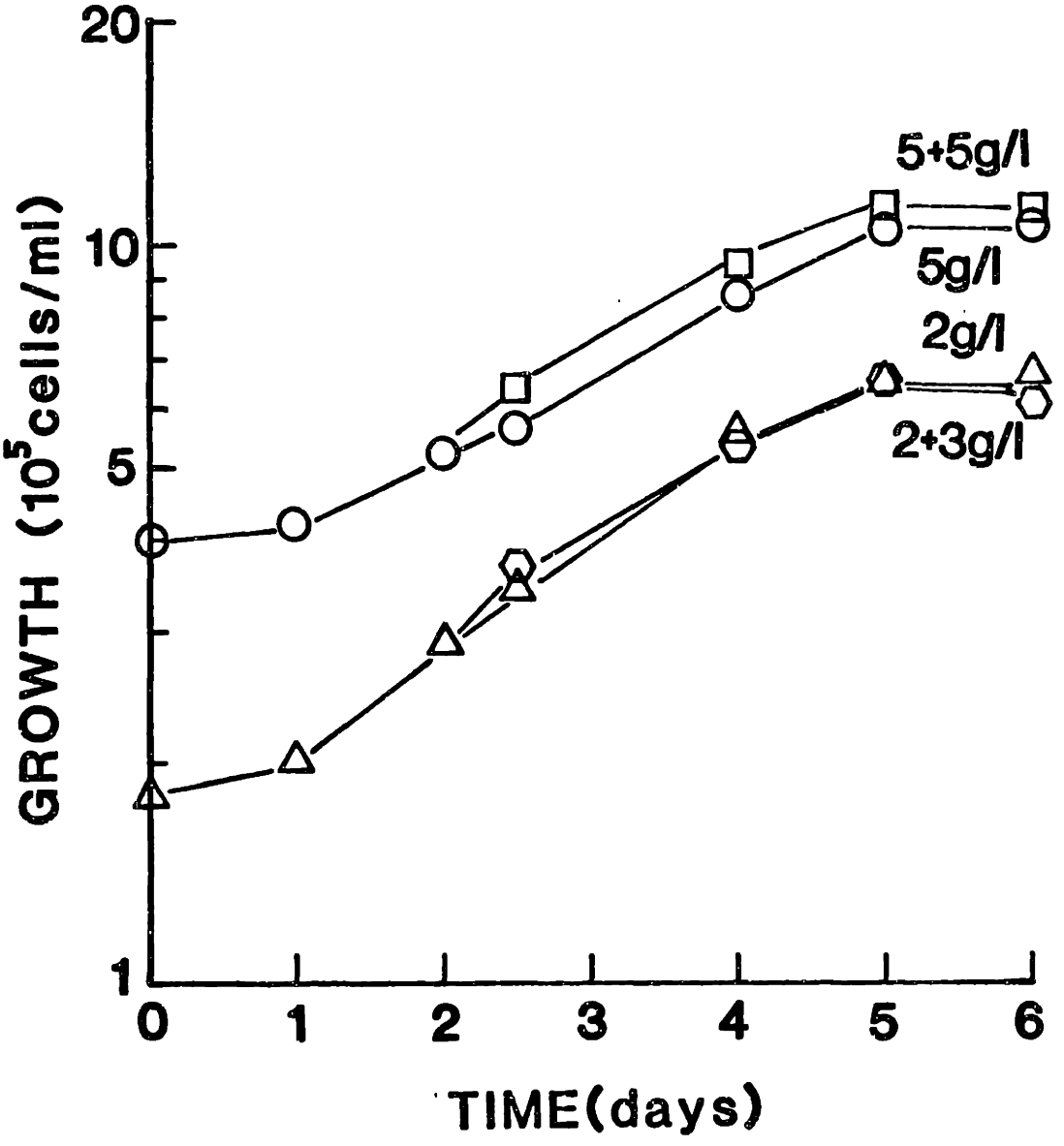
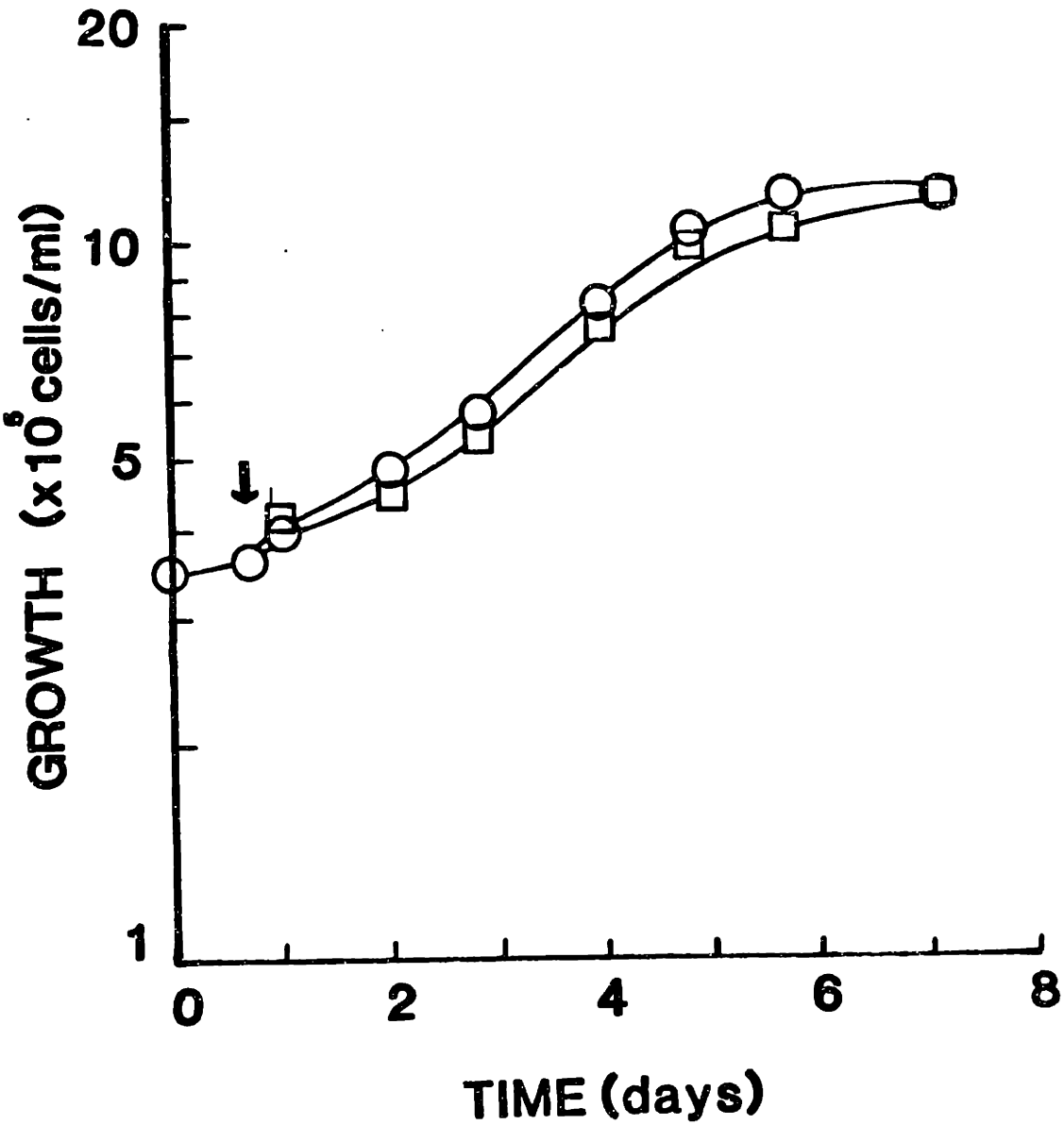

20
10
5
1
GROWTH (10^5cells/ml)
0
1
2
3
4
5
6
TIME(days)
5+5g/l
5g/l
2g/l
2+3g/l

Figure 1-6. Effect of Addition of New Beads to Microcarrier Cultures. Microcarrier concentrations was initially 5 g/l at 16 hr (↓) new microcarriers were added at 10 g/l, thus giving rise a final microcarrier concentration of 15 g/l.


20
10
5
1
GROWTH (x10⁶ cells/ml)
0
2
4
6
8
TIME (days)

and 5.0 g/l. The growth extent decreased with increasing microcarrier concentrations. They thus concluded that the optimum microcarrier concentration was 1 to 2 g/l. This conclusion is inconsistent with the results of Figures 1-5 and 1-6 which strongly suggest that microcarrier concentrations per se do not affect cell growth.

Mered et al. proposed three possibilities to account for the decrease in cell density at higher microcarrier concentrations: (1) increased bead collision leading to increased shear forces , (2) toxic effect(s) of the microcarriers, (3) limitation of nutritional factor(s). The results shown in Figures 1-4 and 1-5 can be used to address the three possible explanations raised in their studies.

Since the addition of new microcarriers to a microcarrier culture did not affect the growth rate or extent, it is unlikely that the increased microcarrier concentration affects the growth through increased collision frequency. The same argument can be applied to the possibility of growth inhibition by a toxic effect due to higher microcarrier concentration.

In the study reported by Mered et al., 50% of the medium was changed every two to three days regardless of the microcarrier and cell concentrations. Because inoculum cell density (cells/cm^2 microcarrier surface) was maintained constant while microcarrier concentrations were varied, the cell concentrations and thus the rates of nutrient consumption and metabolite accumulation also varied. It is probable that the optimum microcarrier concentration reported by them of 1 to 2

g/l, a relatively low concentration, was an artifact caused by nutritional factors. Since nutrient consumption rates were not reported in the study of Mered et al., the posibility that nutrional factor(s) was the growth-limiting factors in their study cannot be excluded. However, in the study which will be presented below, it was shown that with sufficient nutrient supply cells could grow to the maximum growth extent regardless of the microcarrier concentration.

The results presented thus far support the notion that the initial cell distribution on microcarriers affects the subsequent growth. In order to examine the effect of microcarrier concentration independent of cell distribution, it was desirable to compare the growth kinetics of two cultures having identical distributions of cells on microcarriers. This was achieved by allowing cell attachment to occur under identical conditions, and later varying the microcarrier concentrations.

Cultures with a microcarrier concentration of 5g/l and inoculum concentration of 3.5×10^5 cells/ml were prepared in 4 separate spinner flasks. One of the spinners was employed as the control. After cell attachment, the microcarriers from the three other spinners were pooled and two thirds of the microcarrier-free medium was withdrawn to increase the microcarrier and cell concentrations three fold. The pooled microcarrier culture can thus be assumed to have the same cell distribution as the control. At the higher microcarrier concentration, more frequent medium changes were performed (as indicated by the arrows in Figure 1-7) to avoid any possible

nutrient limitations as might have occurred in the study of Mered et al. The growth kinetics of the culture containing 15g/l microcarriers were compared to those of the control. The results from these experiments are shown in Figure 1-7.

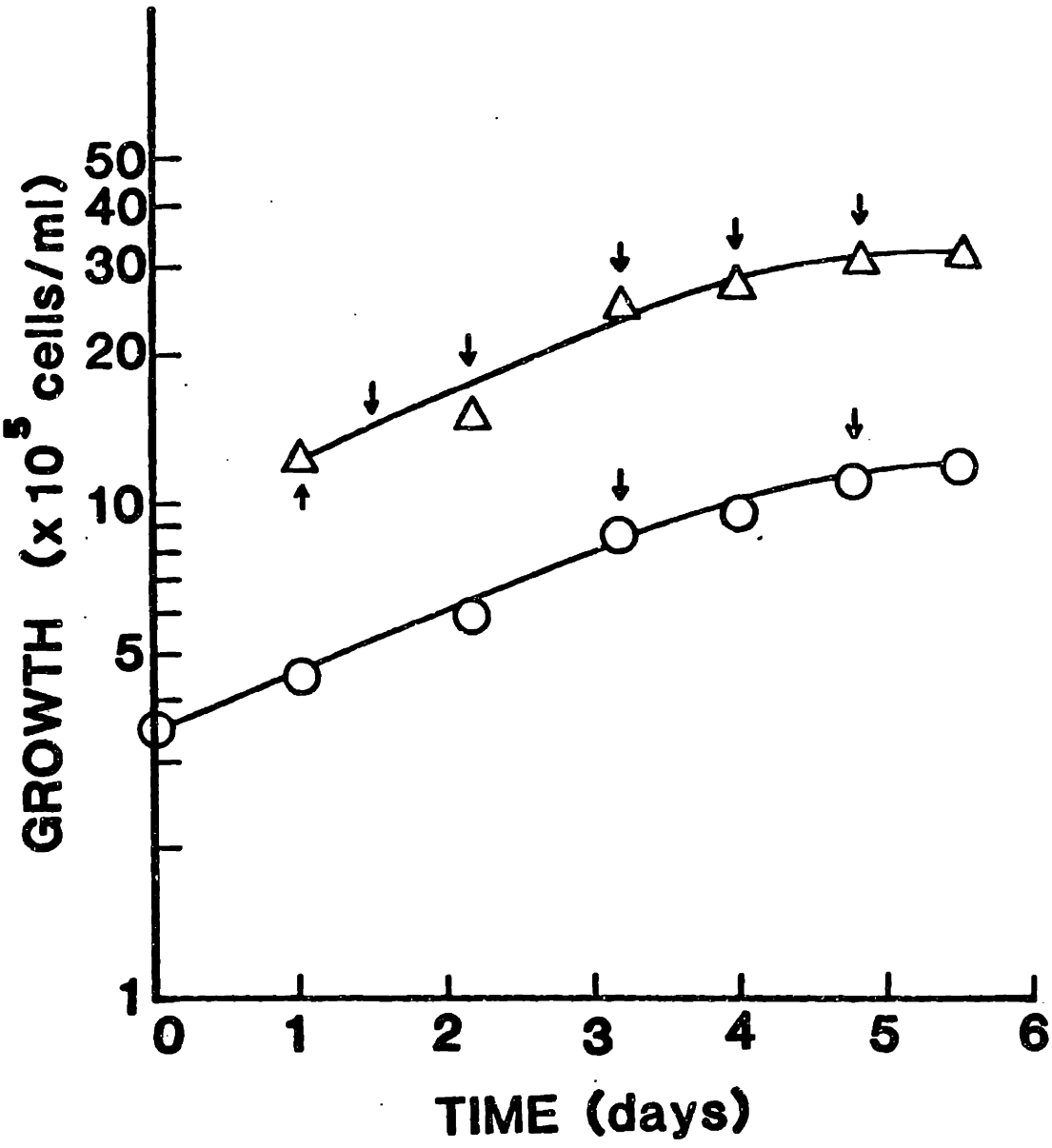
It can be seen from the results in Figure 1-7 that the growth rates for these two cultures are very similar. The final cell concentration in the pooled culture was three times that of the control. This was to be expected since the total surface for cell growth was increased three-fold (5g/l to 15g/l microcarrier concentration).

The results presented in this section demonstrate that microcarrier concentrations per se do not affect cell growth and suggest strongly that cell distribution is critical for achieving a desirable growth rate and growth extent.

1.1.3 THEORETICAL ANALYSIS OF CELL DISTRIBUTION ON MICROCARRIERS

The results presented in the previous section are consistent with the argument that, under the cultivation conditions used, the microenvironment rather than the bulk medium is the critical factor affecting cell growth at low inoculum concentrations. The results also support the notion that the growth microenvironment is affected by the cell distribution, which is a function of inoculum concentration. If one assumes that cell attachment is a probabilistic phenomenon, sparsely

Figure 1-7. Growth of FS-4 Cells at High Microcarrier Concentration. (o):control, 5 g/l of microcarriers; (▵): at 24 hr (↑) three spinners were combined to triple the microcarrier and cell concentrations.


GROWTH (x10^5 cells/ml)
50
40
30
20
10
5
1
0
1
2
3
4
5
6
TIME (days)

inoculated microcarriers would be present in a culture even when inoculated at a high cell concentration. Conversely, densely populated microcarriers could also occur in a culture that received a relatively low inoculum concentration. Therefore, a heterogeneous population can arise as a result of uneven cell distribution. If one further assumes that growth is affected by the microenvironment associated with the cell distribution on microcarriers, then it follows that cells on densely populated microcarriers will grow normally until confluence, whereas the growth of cells on sparsely populated microcarriers will be impeded, whether a culture is inoculated at a high or at a low inoculum concentration. In other words, cells on different microcarriers may behave differently even though they are in the same culture. The reduced growth rate and lower growth extent observed at low inoculum concentrations could arise from a larger fraction of sparsely populated microcarriers on which cell growth is impeded.

Biochemical characterization of the environmental and physiological parameters could lead to the definition of factors critical for cell growth at low inoculum concentration. However, since the growth medium used in this study is highly complex, biochemical characterization would be difficult if not possible. An alterate approach was therefore taken to model the cell growth on microcarriers. This model was then used to identify the important parameters in achieving high extents of growth or high mutiplication ratios.

This proposed model was formulated as follows:

When cells are inoculated into a population of microcarriers, they will attach to the microcarriers in a random fashion following some type of distribution function;

After cell attachment, a critical number of cells per microcarrier is required for normal growth to occur; normal growth is defined here as growth at the maximum growth rate to the maximum extent of growth (i.e. to confluency on the microcarrier surface);

Cells on all of the microcarriers whose initial number of cells is greater than the critical number will grow normally to confluency;

Cells on those microcarriers whose initial number of cells is not greater than the critical number will exhibit impeded growth and will not attain confluency as rapidly as cells exhibiting normal growth.

The discreteness of the microcarrier surface has an important effect on cell growth kinetics. For normal diploid fibroblasts, the characteristic social behaviors, such as parallel cell alignment and avoidance of cell contact, do not extend across microcarriers. Nor can cells avoid overcrowding on one bead by migrating to another. To develop a mathematical model for cell growth on microcarriers, it is necessary to analyze the growth kinetics for cells on individual microcarriers.

In order to develop the mathematical model, a number of assumptions are made. It is assumed that upon the inoculation of cells into the microcarrier suspension, complete mixing is achieved instantaneously. All of the cells in the inoculum are assumed to be separated as individual cells (i.e., no aggregrates of several cells). Furthermore, it is assumed that the attachment of cells to microcarriers follows some integral, discrete distribution function. One of the functions often used to predict the frequency distribution of integral functions is the Poisson distribution. Therefore, although there exist many different types of distribution functions, it was decided to examine first the Poisson distribution. This function was then tested experimentally to determine its adequacy in describing the observed behavior.

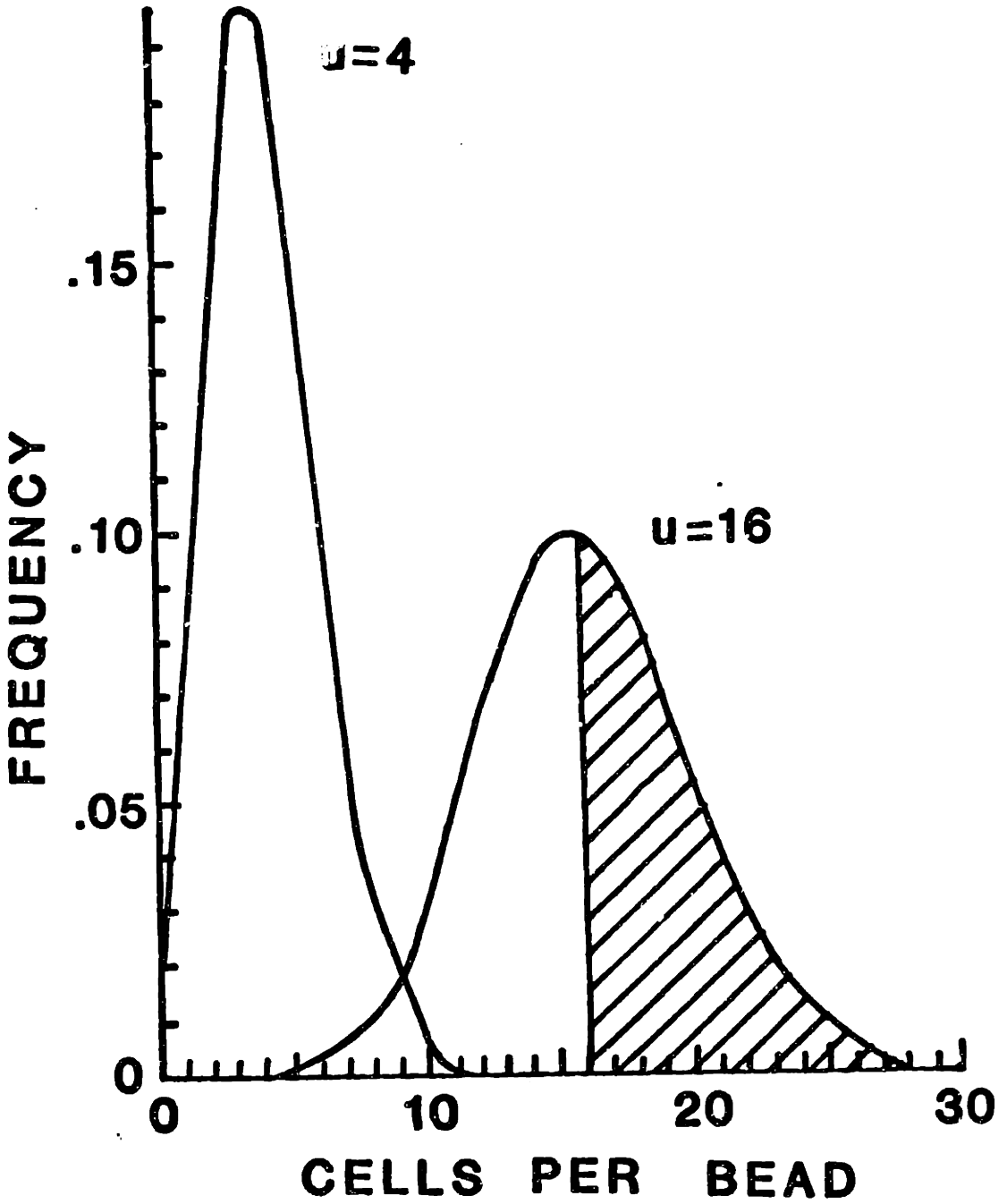
As a first approximation, the microcarriers are assumed to be uniform in size having an identical diameter, D. In a Poisson distribution, if the mean cell number per bead is U, the frequency, f(j), of microcarriers having j cells each will be that shown in equation (1).

$$w(j) = \frac{e^{-U} U^{j}}{j!}$$

(Eq. 1)

Graphical representation of the Poisson distribution is illustrated in Figure 1-8. It can be seen from equation 1 that one of the parameters which differentiates one distribution function from another is the mean cell number per bead (U). To

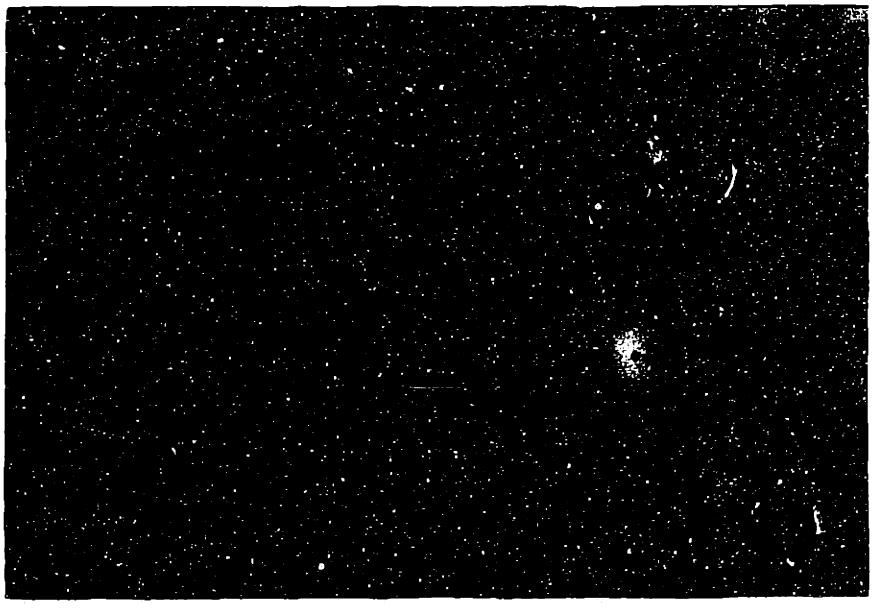
Figure 1-8. Illustrations of Cell Distribution Predicted by the Poisson Function. Microcarriers were assumed to be uniform in size. The average cell number per microcarrier were 4 and 16 respectively.


u=4
u=16
.15
.10
.05
0
FREQUENCY
0
10
20
30
CELLS PER BEAD

illustrate the effect of U on the distribution function, two values of U, 4 and 16, were used in the distributions illustrated in Figure 1-8. The physical significance of a critical number per bead for normal growth can be inferred from the illustration in Figure 1-8. In the case of the mean cell number per bead of 16, if it is further assumed that the critical number of cells per bead is also 16, the fraction of microcarriers having an initial cell number larger than 16 is shown in Figure 1-8 as a shaded area. According to the critical number hypotheses, only those cells on the microcarriers in the shaded area will be able to grow and achieve the desired extent of growth. Those cells in the unshaded area in Figure 1-8 would not attain enough cells for normal growth to occur and therefore would be unable to grow to confluency within the desired time. Extending this analysis of the distribution function to a mean cell number per bead of 4 (U=4), again assuming a critical number of 16,the model predicts that almost none of the cells would grow normally to confluence.

In order to experimentally verify the proposed model, the first step is to verify that the Poisson distribution function is a reasonable model for the cell distribution on microcarriers. The most direct method of obtaining the distribution function of cells on microcarriers is to count the number of cells attached to microcarriers as observed visually through the microscope.For the counting to be accurate, it is necessary to count cells a few hours after inoculation. At this time,the cells are attached but have not yet spread out, such that their more spherical shape and clearer images render direct scoring possible. Also higher

Figure 1-9. Attachment of FS-4 Cells to Microcarriers. FS-4 cells attached to microcarriers two hours after inoculation. The cells were stained with 0.2% crystal violet.



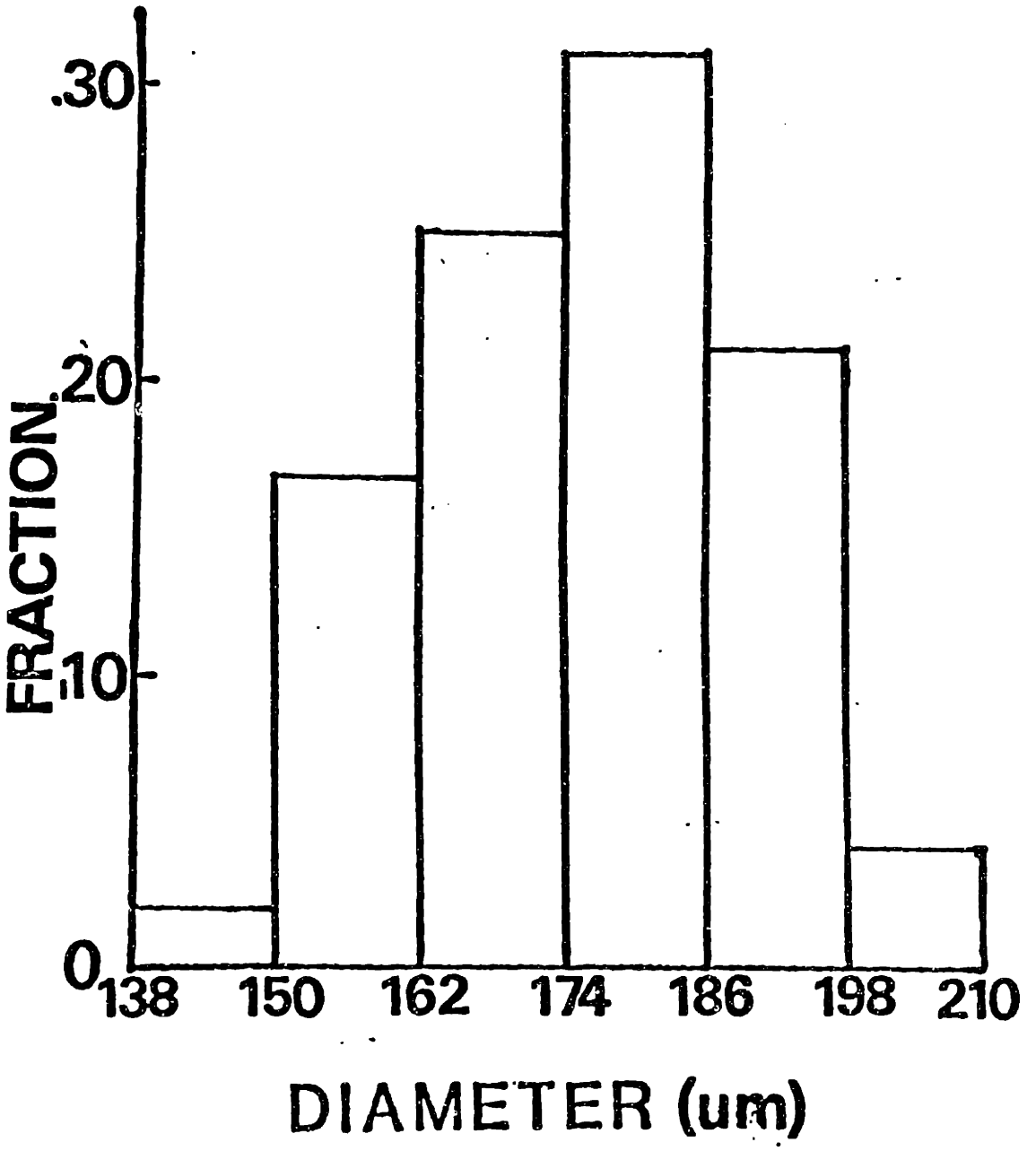
counts due to cell growth following attachment are avoided. Figure 1-9 is a photomicrograph of typical initial cell attachment two hours after inoculation.

Before the experimental verification of the validity of the Poisson distribution function is presented, it should be repeated that the microcarriers are assumed here to be uniform in diameter. This assumption is an approximation since microscopic examination has shown that microcarrier diameter varies. This can be clearly seen in the photomicrograph shown as Figure 1-9. The bead size distribution was measured as described in Materials and Methods. A histogram of the microcarrier size distribution as used in the subsequent analysis is shown in Figure 1-10.

In order to take into account the effect of the microcarrier diameter variation on the cell attachment distribution, it was assumed that the attachment of cells onto any microcarrier is directly proportional to the bead's external surface area. In view of the limited accuracy on the diameter measurements as well as on the microscopic examination of cell numbers, a discrete distribution of microcarrier diameter was used for the following analysis. The microcarriers are divided into i-number of groups, each group having a different diameter. The group denoted as m is referenced to the one having a median diameter of D_m which will be used for subsequent calculations for diameter variations.

In the following analysis, it is assumed that a cell concentration of X cells per ml is used for the inoculation of N

Figure 1-10. Histogram of Diameter Distribution of Microcarriers. The median diameter of the microcarriers used for analysis of the critical number was 185 μ. The microcarriers were suspended in phosphate buffer saline for the measurement of diameter.


.30
.20
.10
0
FRACTION
138
150
162
174
186
198
210
DIAMETER (um)

microcarriers per ml. The weighted mean of cell number per bead for group m of microcarriers can be described by equation 2:

$$U(D_m) = \frac{X}{N\sum_{all\,i} f_i (D_m/D_i)^2}$$

(Eq. 2)

In equation 2, f_i is the fraction of microcarriers having diameter D_i. Based on the reference microcarriers having diameter D_m, the mean cell number per bead, $U(D_i)$ for the group of microcarriers with diameter D_i can be calculated. The expression for $U(D_i)$ is shown below as equation (3).

$$U(D_i) = U(D_m) \times (D_i/D_m)^2$$

(Eq. 3)

The probability, $g_i(j)$ for microcarriers having diameter D_i to acquire j cells can then be calculated by substituting U in equation 1 with the true average cell number per bead, $U(D_i)$.

$$g_i(j) = \frac{e^{-U(D_i)}\, U(D_i)^j}{j!}$$

(Eq. 4)

Since the microcarriers with the diameter D_i constitute only fraction (f_i) of all of the microcarrier population, the frequency of those microcarriers having a diameter of D_i and containing J cells in the entire population is the product of f_i and $g_i(J)$. The actual frequency (F(j)) of all microcarriers having j cells per bead can be obtained by summing the fractions contributed by all group having differenr diameters. The actual frequency of microcarriers having j cells per bead is expressed as equation 5.

$$F(j) = \sum_{i=0}^{I} f_i \, g_i(j)$$

(Eq. 5)

In equation 5, the fraction of microcarriers with diameter D_i can be obtained from the histogram of the diameter distribution function (Figure 1-10). The average cell number per microcarriers $U(D_i)$ can be calculated from equation 3. It should be noted that in order to calculate the value of $U(D_i)$, one must first calculate the reference diameter $U(D_m)$. This reference diameter can be calculated from cell concentration X, and microcarrier concentration, N as shown in Equation 2. Therefore, knowing the inoculum concentration, microcarrier concentration and bead diameter distribution, the cell distribution on microcarriers can now be calculated. The calculated cell distribution can then be compared to that of experimental observation.

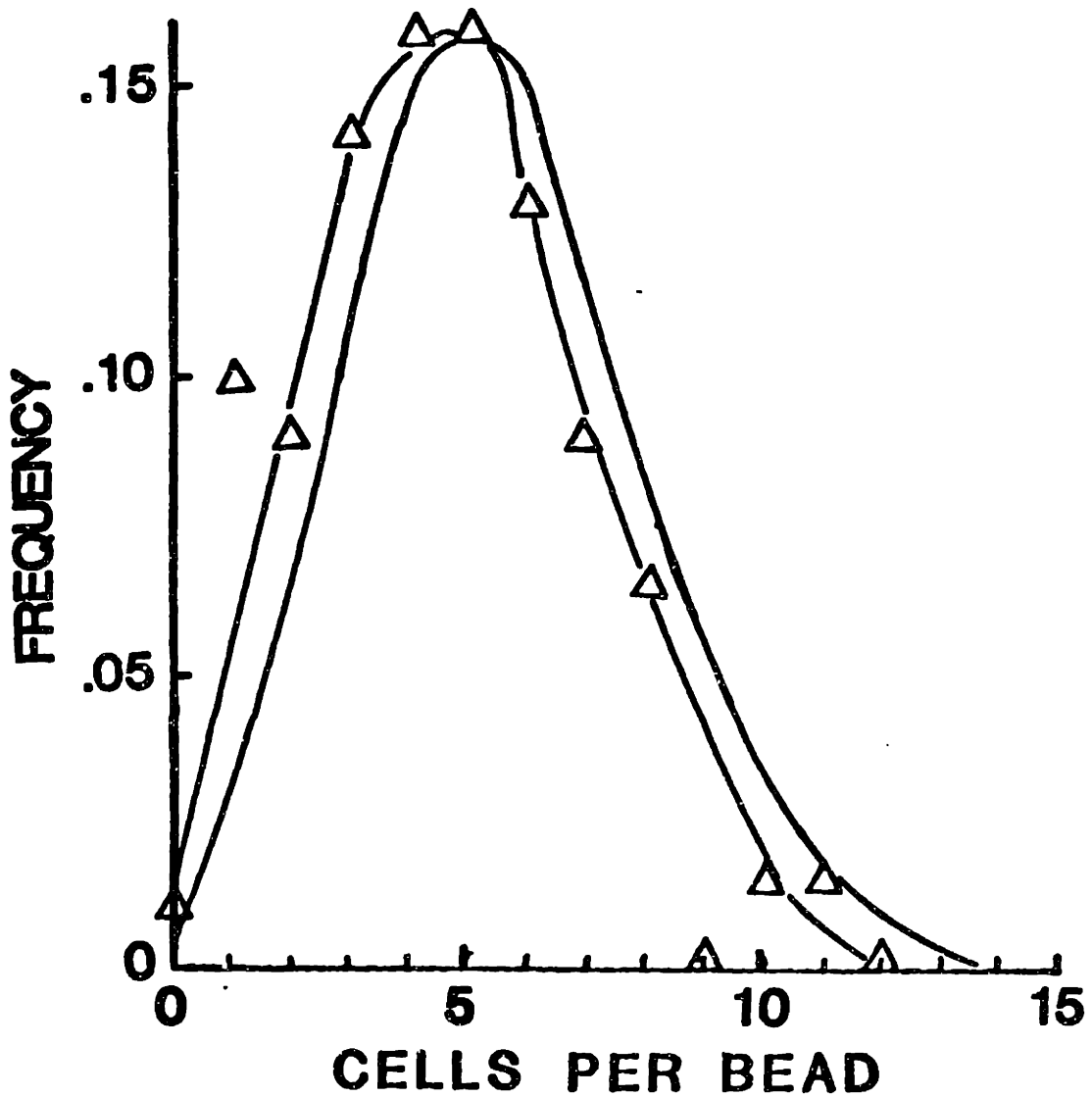
Shown in Figure 1-11 are the cell distribution curves actually observed and that predicted by Poisson distribution after correcting for the bead size distribution. The inoculum concentration used was 1.2×10^5 cells/ml and the microcarrier concentration was 5g/l. The experimentally observed distribution correlated reasonably well to that of theoretical prediction.

The difference in the calculated and experimental cell distributions is mostly likely to be caused by the difficulty of scoring cells accurately using microphotographs. This is because cell distribution on microcarriers in a three dimensional manner. However, photomicrographs can only project cells in a two dimensional fashion leading to errors in the absolute scoring. The result from Figure 1-10 strongly suggests that the cells attach to microcarriers randomly, and the cell distribution on microcarriers can be predicted by Equation 5.

1.1.4 MATHEMATICAL MODELING OF CELL GROWTH ON MICROCARRIERS

In this section the equations derived in Section 1.1.3 will be used to develop a growth model. The model will subsequently be used to simulate cell growth at different conditions to define the critical inoculum cell nummber. In the development of the growth model, cell growth on each individual microcarrier will be examined first. The total cell concentration during growth will then be obtained by the summing the cell density on each of the

Figure 1-11. Comparison of Predicted Cell Distribution and Experimental observation. (____): theoretical prediction; (-Δ-): experimental observation. The microcarrier concentration was 5 g/l and the inoculum cell concentration was 1.2×10^5 cells/ml.


.15
.10
.05
0
FREQUENCY
0
5
10
15
CELLS PER BEAD

microcarriers. In the proposed model, it was postulated that cell growth on any microcarrier is a function of the initial cell number on that microcarrier; a critical number is required for normal growth to occur. However, such a growth rate-inoculum function cannot be obtained until we have a better understanding of the biological characteristics of cell growth on microcarriers. To develop the understanding leading to a growth model, the growth rate was assumed to be a discrete function of the initial cell number on a microcarrier. If the initial cell number on any microcarrier is larger than the critical number, cells will be assumed to grow normally until confluence. On the other hand, if the initial cell number is smaller or equal to the critical number, the growth rate will be assumed to be negligible. The cell number on those microcarriers will thus remain the same as the initial number.

Under optimal conditions, the growth of cells proceeds to confluence. For given culture conditions, the confluent cell density on a per unit surface area basis is constant. The maximum cell concentration, X_{max}, at a given microcarrier concentration, N, is known from the experimental results presented in Section 1.1.1 (Figures 1-1 and 1-3). Therefore, the confluent cell density per microcarrier for a reference group having diameter D_m can be calculated. This is shown as Equation 6.

$$x_{max}(D_m) = \frac{X_{max}}{N\Sigma\, f_i(D_m/D_i)^2}$$

(Eq. 6)

After knowing the confluent cell density of the microcarriers having the diameter D_m, the confluent cell density of microcarriers having diameter D_i can be calculated by Equation 7.

$$x_{max}(D_i) = x_{max}(D_m)(D_i/D_m)^2$$

(Eq. 7)

To develop the equations describing cell growth, focus will first be directed to a simpler situation in which no critical cell number is required. The requirement of a critical cell number will be considered subsequently. It is assumed that cells multiply at a specific growth rate μ until confluence is reached. If the initial cell number on a microcarrier is j, at any time t during the growth stage, the number of cells can be expressed as $je^{\mu t}$. However, on any microcarrier having a diameter D_i, the maximal cell number per bead is the confluent density, $x_{max}(D_i)$ thus, after reaching confluence, the cell number will be $x_{max}(D_j)$ For any microcarrier having a diameter D_i and with j cells initially attached after inoculuation, the cell growth extent, $x(D_i, j, t)$, can be expressed by Equation 8.

$$x(D_i,j,t) = je^{\mu t} \quad \text{if } je^{\mu t} < x_{max}(D_i)$$

$$x(D_i,j,t) = x_{max}(D_i) \quad \text{if } je^{\mu t} > x_{max}(D_i)$$

(Eq. 8)

In Equation 8, a step change in growth rate from μ to 0 is assumed when cells reach confluence. However, for normal diploid cells, the actual growth rate decreases gradually as confluence is approached. The dependence of growth rate on the extent of growth must be taken into account in the mathematical model. To experimentally determine the dependence of growth rate on the extent of confluence, the nonuniformity of microcarrier diameters and uneven cell distribution on microcarriers must be avoided. To simulate growth kinetics of cells evenly distributed on microcarriers of uniform diameter, Petri dishes 10 cm in diameter were used to cultivate FS-4 cells. The medium was changed every other day in order to prevent nutrient limitations. The growth kinetics under these conditions are shown in Figure 1-12. After a short lag period, cells grew exponentially, but the growth rate decreased appreciably as cells approached confluence. The specific growth rate was calculated from the growth kinetics shown in Figure 1-12. These growth rate were expressed as a function of the extent of confluence and are shown in Figure 1-13. This experimentally derived function should be incorporated into the equation for cell growth (Eq. 8). Thus,

Figure 1-12. Simulation of Cell Growth on Microcarriers of Infinite Diameter. 10 cm Petri dishes were used to simulate the growth of FS-4 cells uniformly distributed on microcarriers of infinite dimeter.

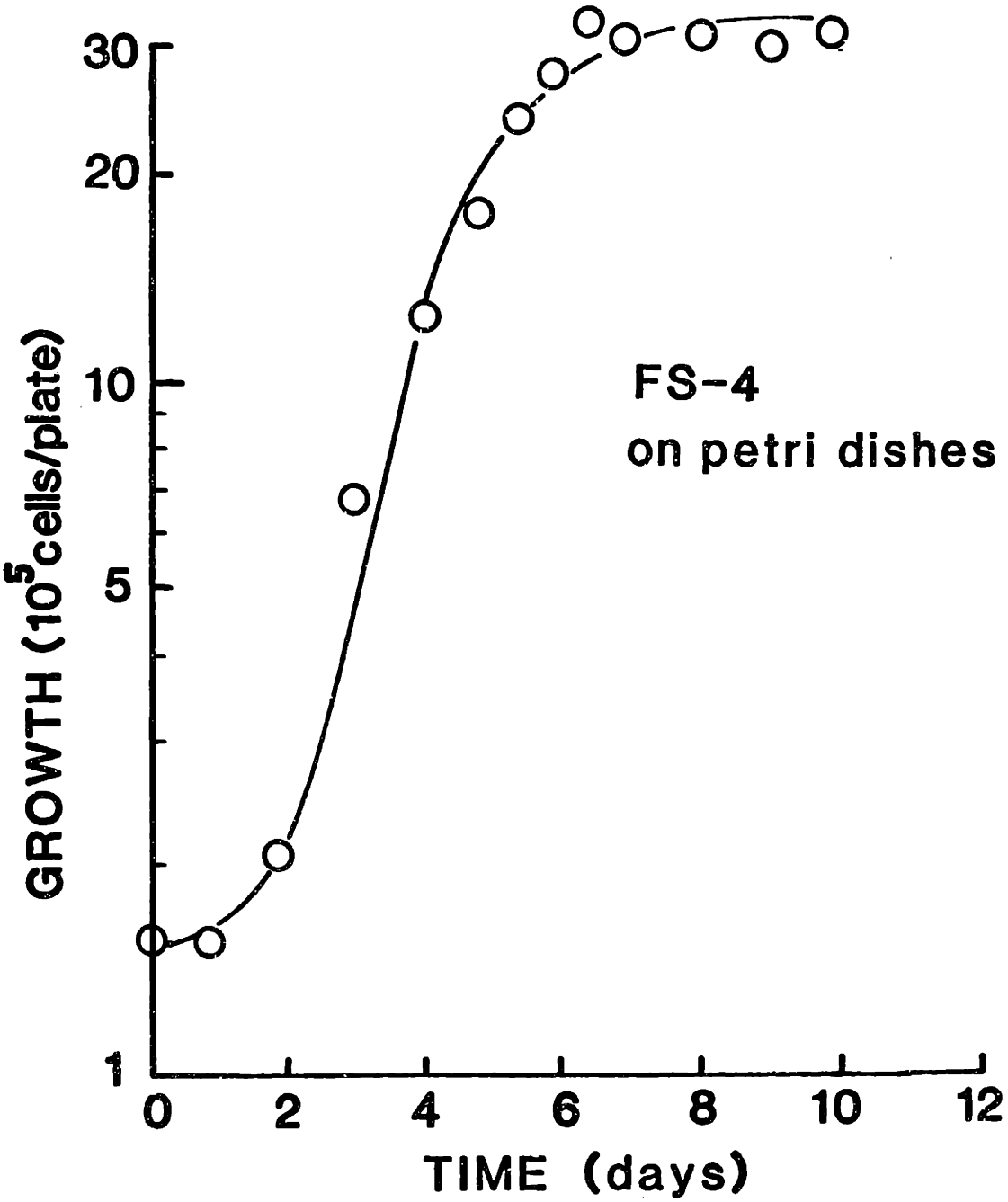
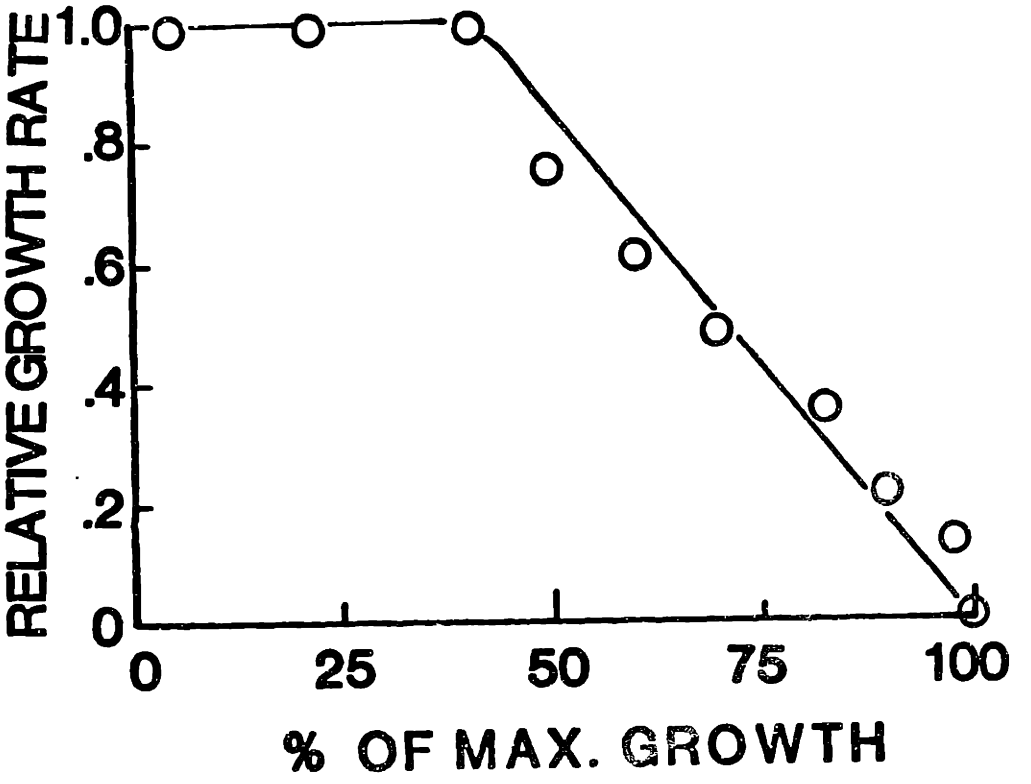

30
20
10
5
1
GROWTH (10^5cells/plate)
FS-4
on petri dishes
0
2
4
6
8
10
12
TIME (days)

Figure 1-13. Relationship of Growth Rate and the Extent of Confluence. The growth rate of FS-4 cells on Petri dishes from Figure 1-12 was expressed as a function of the percentage of the maximal extent of cell growth.


RELATIVE GROWTH RATE
1.0
.8
.6
.4
.2
0
0
25
50
75
100
% OF MAX. GROWTH

the correct expression for cell growth on a microcarrier can be obtained by substituting μ in Equation 8 with $\mu(x)$. This is shown in Equation 9.

$$x(D_i,j,t) = je^{\mu(x)t} \quad \text{if } je^{\mu(x)t} < x_{max}$$

$$x(D_i,j,t) = x_{max} \quad \text{if } je^{\mu(x)t} \geq x_{max}$$

(Eq. 9)

Equation 9 describes cell growth initiated with j cells on a microcarrier of diameter D_i. As mentioned previously, the requirement of a critical cell number was not considered in the development of Equation 9. To account for this requirement, it was assumed that the critical cell number for a given microcarrier is proportional to the external surface area of that microcarrier. The critical cell number per microcarrier for microcarriers having diameter D_i, can again be calculated from that for the reference microcarriers. This is shown in Equation 10.

$$x_c(D_i) = x_c(D_m)(D_i/D_m)^2$$

(Eq. 10)

In Equation 10, $x_c(D_m)$ is the critical cell number of the microcarrier reference with respect to the microcarriers with median diameter, D_m.

The growth extent, $x(D_i,j,t)$, for any microcarrier with diameter D_i and at time t, is affected by the initial cell number j which it has acquired at inoculation. According to the assumptions made above, if the initial cell number j is smaller than or equal to the critical number, x_c, then cell growth is negligible and the cell number will remain equal to j. If the initial cell number j is larger than the critical number x_c, then cells will grow normally as described by Equation 9. The equation for cell growth is then modified as shown in Equation 11.

$$x(D_i,j,t) = j \qquad \text{if } j \le x_c$$

$$x(D_i,j,t) = je^{\mu(x)t} \qquad \text{if } j > x_c \text{ , } je^{\mu(x)t} < x_{max}$$

$$x(D_i,j,t) = x_{max} \qquad \text{if } j > x_c \text{ , } je^{\mu(x)t} \ge x_{max}$$

(Eq. 11)

The equation for cell growth on individual microcarriers having been derived, the cell concentration per unit volume can be obtained by the summation of cell number on all of the

microcarriers. The probability of a microcarrier having diameter D_i acquiring j cells after inoculation is $g_i(D_i)$ as shown in Equation 4. The fraction of the microcarriers having diameter D_i is f_i and the number of microcarriers per unit volume is N. Thus, the concentration of microcarriers having diameter D_i and initiated with j cells is $Nf_ig_i(D_i)$. The cell concentration contributed by those microcarriers at any time t is thus the product of $Nf_ig_i(D_i)$ and $x(D_i,j,t)$. The actual cell concentration X(t) at time t, is the summation of the contributions by all microcarriers having different diameters D_i and initial cell densities j. This is shown in Equation 12.

$$X(t) = \sum_{all\ i} \sum_{all\ j} Nf_ig_i(D_i)\ x(D_i,j,t)$$

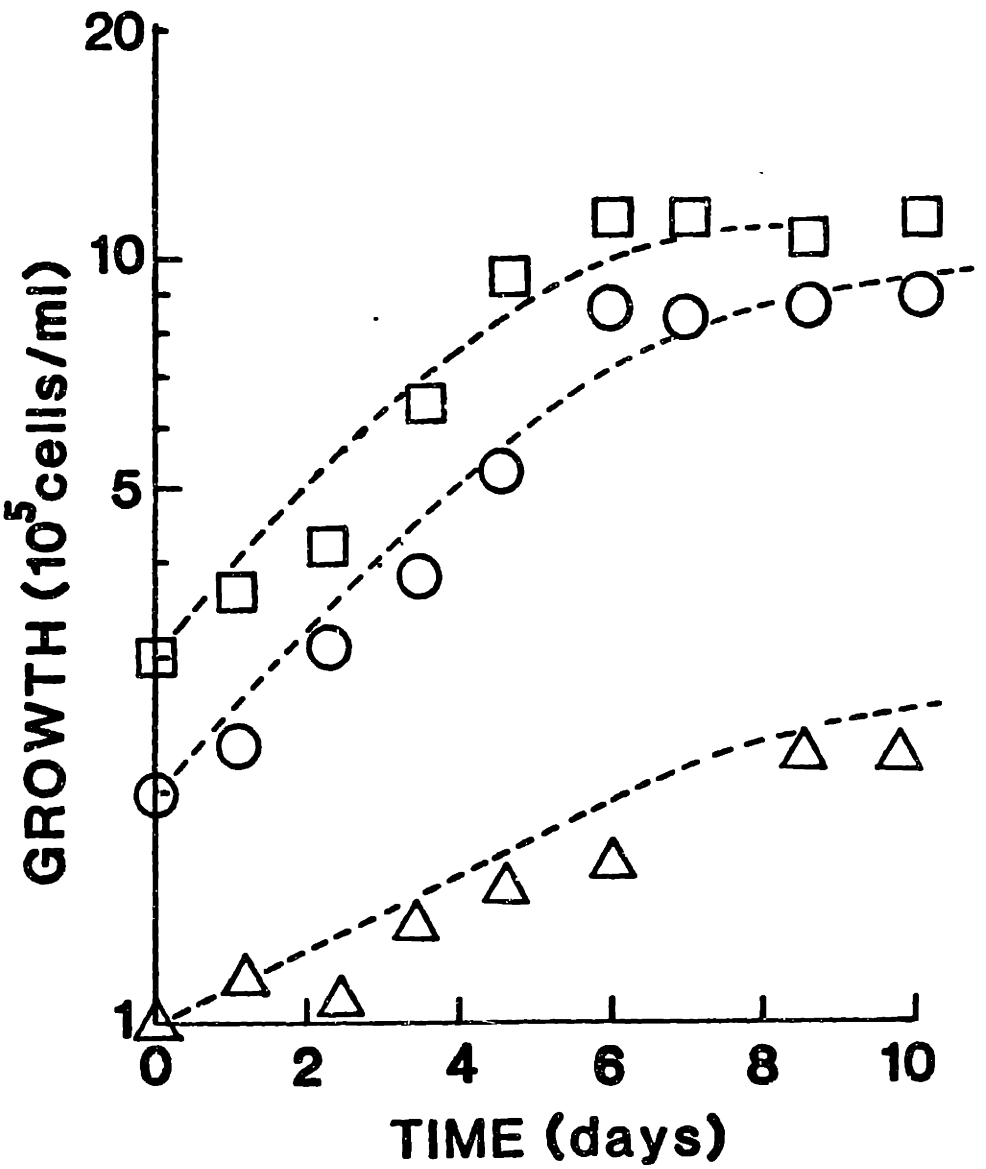
(Eq. 12)

In applying Equation 12 to predict cell concentration, X(t), it is necessary to know the inoculum cell concentration,X(0), the microcarrier concentration, N, and the critical cell number for the reference microcarrier, $x_c\ (D_m)$, as well as the microcarrier diameter distribution. Except for the critical cell number, the values of these parameters can all be established under the experimental conditions. If the critical cell number per microcarrier, $x_c(D_m)$, is specified, then cell growth can be simulated. Using this growth model, the growth of FS-4 cells for different inoculum concentrations was simulated with various

values for the critical cell number. The simulated growth kinetics were compared to experimental results. In this simulation, the critical cell number, $x_c(D_m)$, for microcarriers having the median reference diameter was set equal to six cells per microcarrier. The experimentally observed growth kinetics compared very well with those simulated with the model (Figure 1-14). Therefore, based on the proposed model of cell growth, the critical cell number was determined to be six for microcarriers of the median (reference) diameter.

The requirement of a minimum inoculum density for mammalian cell culture has been reported (Eagle and Piez, 1958, 1962). In a more recent study, Kaighn et al. (1981) reported that the growth rate of four carcinoma cell lines in serum-free medium was population density dependent. However, in these and other studies, such phenomena seemed to be due to diffusible factors. Thus, the dependence was probably on a per unit volume basis. In the results presented in this thesis, the inoculum requirement has been attributed to a dependency on a per unit surface area basis. A reduced growth rate and a lower growth extent was observed when the inoculum cell concentration was reduced. This has been attributed to impeded growth on those microcarriers which did not acquire enough cells after inoculation. In Section 1.1, it was reasoned that the inoculum dependency was due to the requirement of a permissive microenvironment on the microcarriers. The determining characteristics of the microenvironment could be caused by direct, tactile signals from neighboring cells. Alternatively, they could be related to a

Figure 1-14. Identification of the Critical Number for Cell Growth. Cell growth at different inoculum concentrations as observed experimentally (○ , △ , □) and as predicted by the model, assuming a critical number of six cells per microcarrier (------).


20
10
5
1
GROWTH (10^5cells/ml)
0
2
4
6
8
10
TIME (days)

requirement of the localized accumulation of conditioning factors. This could be the consequence of partitioning or a barrier to diffusion between the microcarriers and the bulk medium. Later in this thesis, it will be shown that the critical cell number can be reduced by the use of a better medium for clonal growth, thus suggesting a mechanism related to diffusible molecules. However, the mechanism of direct cell contact could not be ruled out even though its involvement in growth control at low inoculum cell density has not been proven.

In the model of cell growth on microcarriers presented above, it was assumed implicitly that contact inhibition of cell growth is solely effected by "spatial crowdedness" and not by diffusible growth inhibitory factors. In other words, cells on confluent microcarriers do not secrete a diffusible molecule(s) or do not secrete such a substance(s) to an extent sufficient to affect growth rate on less densely populated microcarriers. The validity of this assumption remains to be tested. Voss et al. (1982) reported that a protein of molecular weight 3,000 was secreted by 3T3 cells when they reached confluence. This low molecular weight protein caused growth inhibition when it was added to a sparse population of cells. The inhibitory effect was reported to be more significant in serum-free medium, since serum contains a substance(s) which offsets the inhibitory effect. If such a density-dependent growth-inhibitory molecule exists in microcarrier culture, it will be important to define the effect of such a molecule on the growth kinetics.

The critical cell number model is somewhat simplified. It assumes that growth is affected by inoculum cell density in an all or none fashion, while, in reality, the growth rate may not be a step function but rather a continuous function of the inoculum cell density. Nevertheless, this model can serve as a foundation for the further analysis of cell growth on microcarriers.

1.2 INCREASE OF MULTIPLICATION RATIO ON MICROCARRIERS

1.2.1 IMPROVEMENT OF CELL GROWTH AT LOW INOCULUM CONCENTRATION

To improve cell growth at low inoculum concentrations, at least two possible approaches can be taken. One is to alter the cell distribution on microcarriers, thus allowing a larger fraction of the microcarriers to acquire enough cells to grow. The second is to improve the environmental conditions, in particular nutritional factors, in order to reduce the critical cell number. As shown in Section 1.1, the critical cell number required for normal growth leading to confluency was found to be six cells per microcarrier.

In order to devise a method to advantageously alter cell distribution on microcarriers, an analysis of this distribution is necessary. Cell distribution on microcarriers can be predicted by the Poisson distribution function. The shape of a Poisson distribution curve is determined by its shape parameter which is the mean cell number per microcarrier. At a given inoculum cell concentration (X), the mean cell number per bead (U) is inversely proportional to the number of microcarriers per unit volume (N). The number of microcarriers required to provide a specified surface area is dependent on the microcarrier diameter (D). Thus, a change in the microcarrier diameter results in the alteration of the cell distribution on the

microcarriers. The effect of the microcarrier diameter on the fraction of microcarriers which acquire enough cells for growth is analyzed below. For purposes of comparison, microcarriers of a given group are assumed to be uniform in diameter. Two different microcarriers (diameter D_1 and D_2) will be used for this illustration. It is assumed that the same inoculum concentration (X) is to be used in both cases. The multiplication ratio was defined previously as the ratio of the maximum growth extent (cells per ml) to the inoculum concentration. In order to achieve the same multiplication ratio for different sizes of microcarriers, the growth surface area per unit volume (A) must be maintained equal. Thus, the number of microcarriers per unit volume will be inversely proportional to the square of the diameter. This relationship is shown as Equation 13.

$$A_1 = A_2$$

$$N_1 D_1^2 = N_2 D_2^2$$

$$N_1/N_2 = (D_2/D_1)^2$$

(Eq. 13)

For a constant inoculum cell concentration, X, the mean cell number per microcarrier is then proportional to the square of the diameter as shown in Equation 14.

$$U(D_1) = X/N_1$$

$$U(D_2) = X/N_2$$

$$U(D_1)/U(D_2) = (D_1/D_2)^2$$

(Eq. 14)

The frequency, w(j), of microcarriers which will acquire j cells at inoculation is shown in Equation 1 from Section 1.1.3.

$$w(j) = \frac{e^{-U} U^{j}}{j!}$$

(Eq. 1)

The cumulative frequency, P, of those microcarriers which have not acquired enough cells for growth to occur is the sum of the frequencies of fractions whose initial cell number j is smaller than or equal to the critical number, x_c. The cummulative frequency, P, is shown as Equation 15.

$$P = \sum_{j=0}^{x_c} \frac{e^{-U} U^{j}}{j!}$$

(Eq. 15)

The critical cell number per bead is assumed to be proportional to the surface area and presented as Equation 16.

$$x_c(D_1)/x_c(D_2) = (D_1/D_2)^2$$

(Eq. 16)

In order to reduce the cumulative frequency, P, the dependence of P on microcarrier diameter must first be defined. The first derivative of P with respect to the mean cell number per microcarrier, U(D), is shown as Equation 17.

$$\frac{dP}{dU} = \frac{1}{2\alpha D} \frac{dP}{dD} = \sum_{j=0}^{x_c} \frac{e^{-U} U^j}{j!} (-U+j)$$

(Eq. 17)

where α is a constant. In Eq. 17 both the mean, U, and the critical number, x_c, are affected by changing microcarrier diameter. Thus, both the number of terms to be summed and the value of each of those terms change with diameter. Eq. 14 and Eq. 16 show that both the critical number and the mean are proportional to the diameter squared. The combination of these two equations, the relationship between the critical cell number per bead and mean number on cells per bead, is shown as Eq. 18.

$$x_c = kU$$

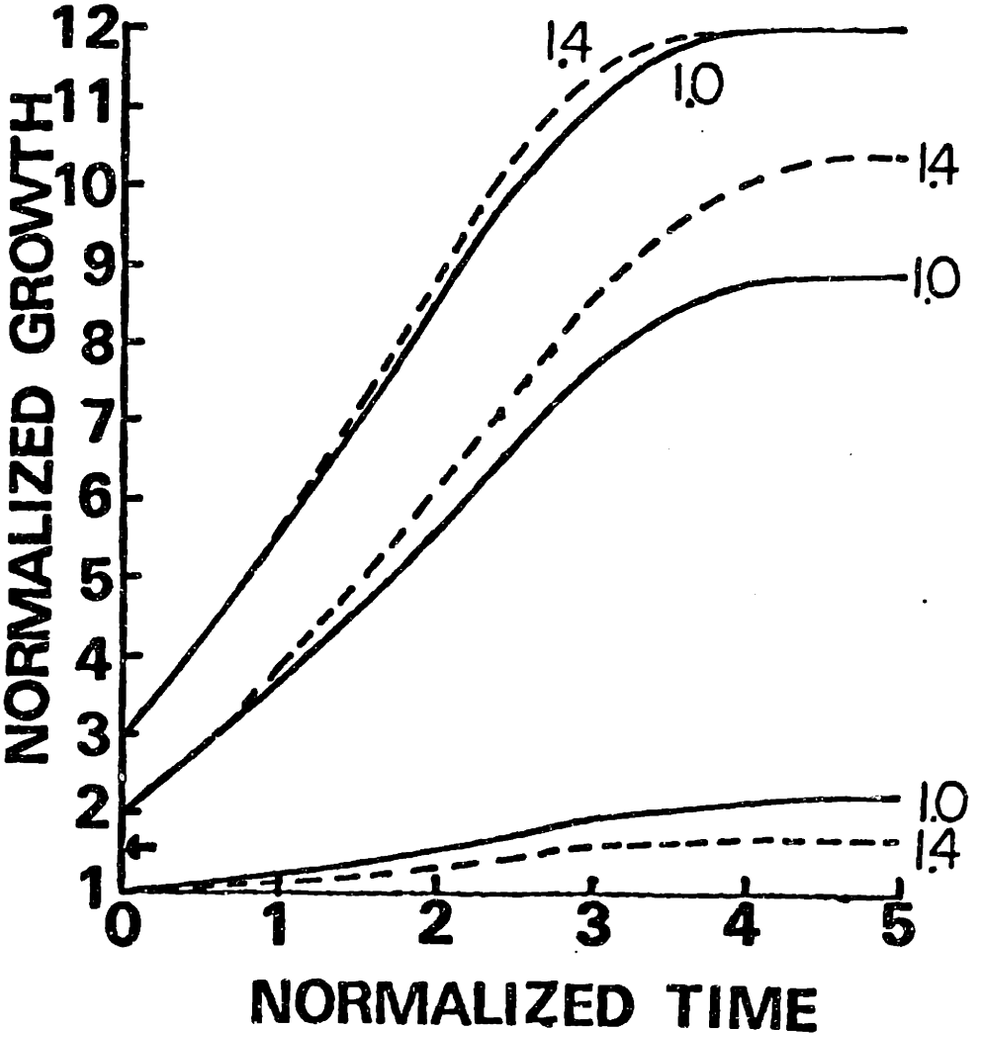
(Eq. 18)

Substituting Eq.18 into Eq. 17, it can be seen that the derivative dP/dD always has a negative value whenever the

critical number, x_c, is smaller than the mean, U. Under these circumstances, the cumulative frequency, P, of microcarriers which have not acquired enough cells for growth is always a decreasing function of the diameter. Therefore, the fraction of microcarriers which have acquired enough cells to allow growth will always increase as microcarrier diameter is increased provided the average cell number per bead after inoculation is greater than the critical number.

Although increasing the microcarrier diameter can increase the fraction of microcarriers which have growing cells, its effectiveness in increasing the multiplication ratio varies with different values of the critical number as well as the inoculum concentration. To illustrate the effects of microcarrier diameter, the growth model was used to simulate cell growth on microcarriers having relative diameters of 1.0 and 1.4. For the purposes of this illustration, the microcarriers were assumed to be uniform in size, and the confluent cell density and the critical cell number were assumed to be proportional to the surface area. For microcarriers having a relative diameter of one, the critical cell number and the confluent cell density were assumed to be 6 and 50 cells per bead respectively. The graphic simulations are shown in Figure 1-15. The three solid lines in Figure 1-15 represent the simulated growth kinetics for values of the average cell number per bead at inoculation of 4, 8 and 12. For microcarriers with relative diameter 1.4, the critical number per microcarrier was assumed to be 12 and the confluent cell density 98 cells per bead. The simulated growth kinetics for

Figure 1-15. Prediction of the Effect of Microcarrier Diameter. (___): microcarriers of relative diameter one and with a critical cell number of 6; (---): microcarriers of relative diameter 1.4 and with a critical number of 12. The total growth surface area was equivalent to that of 5 g/l regular microcarriers. The maximal extent of growth was thus 1.2×10^5 cells/ml.

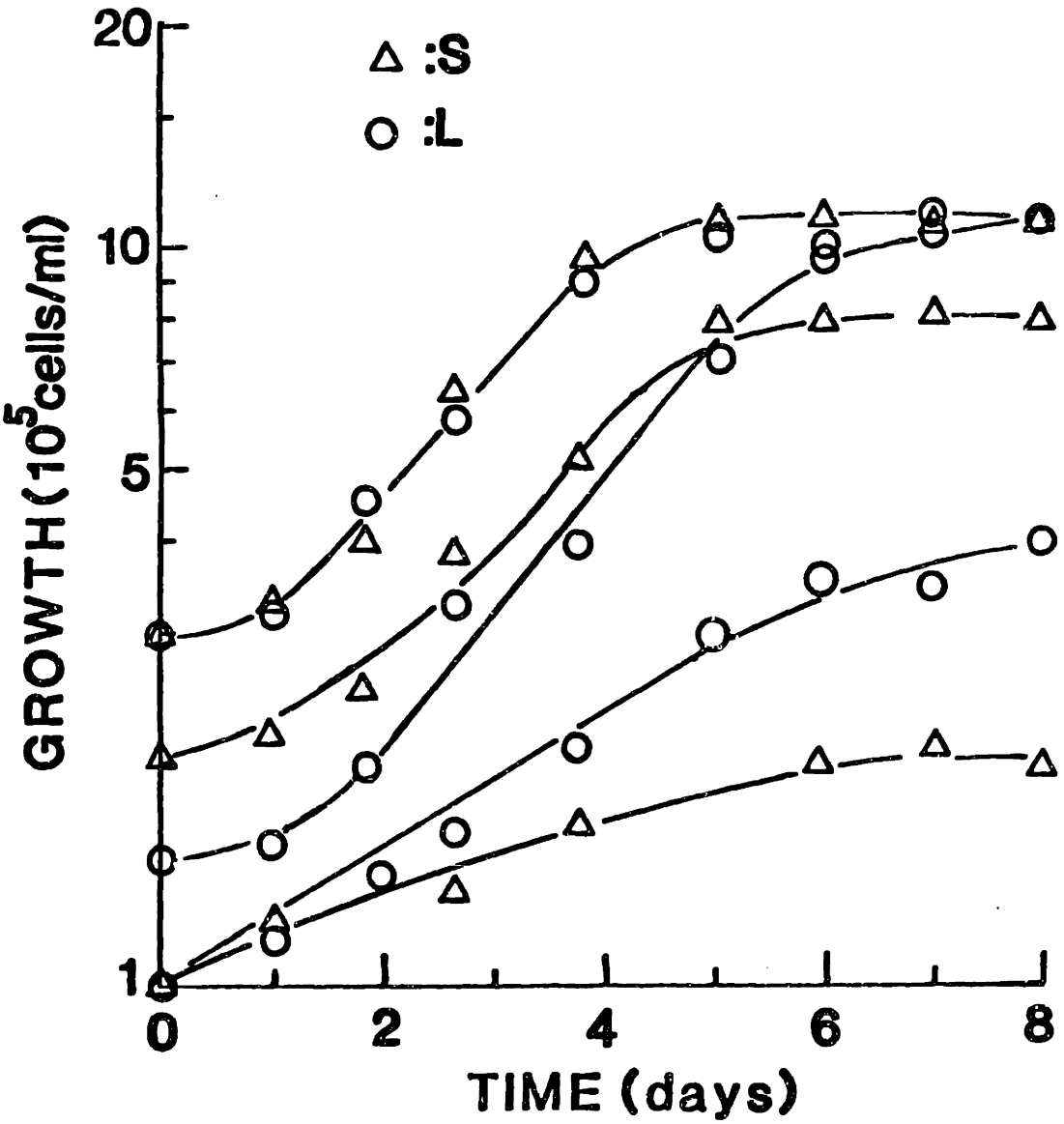

NORMALIZED GROWTH
12
11
10
9
8
7
6
5
4
3
2
1
0
1
2
3
4
5
NORMALIZED TIME
1.4
1.0
1.4
1.0
1.0
1.4

microcarriers having relative diameter 1.4 are shown as broken lines in Figure 1-15. The critical cell number is indicated with an arrow in this Figure. When the inoculum cell concentration is larger than the critical number, the microcarriers of larger diameter show improved cell growth; whereas, at inoculum concentrations lower than the critical number, increasing bead diameter has an adverse effect on cell growth.

From the analysis presented in Section 1.1, the critical cell number for microcarriers with diameter 185μ was shown to be six cells per microcarrier. It is therefore predicted by this model that, at a microcarrier concentration of 5 g/l, when the inoculum concentration is larger than 1.7×10^5 cells/ml, growth would be improved by increasing microcarrier diameter.

An experiment was carried out to test this prediction. Microcarriers with a median diameter of 265 μ were used along with the microcarriers having a median diameter of 185 μ. The concentration was 5 g/l for the smaller microcarriers (185 μ) and 7 g/l for those with a larger median diameter (265 μ). Under these conditions the same surface area per unit volume was maintained. Cells were inoculated at different concentrations and the growth kinetics are shown in Figure 1-16. Cells grew normally on both sizes of microcarriers at high inoculum concentrations. This suggests that the increase in the microcarrier diameter from 185 μ to 265 μ did not alter the surface characteristics for cell growth. At reduced inoculum concentrations, cell growth was improved by the use of microcarriers having a median diameter 265 μ. It was predicted

Figure 1-16. Effect of Microcarrier Diameter on Cell Growth at Different Inoculum Concentrations. (△): microcarriers with median diameter 185 μ at 5 g/l; (○): microcarriers with median diameter 265 μ at 7 g/l. Medium: DME supplemented with 5% FCS.


20
10
5
1
△ :S
○ :L
GROWTH (10^5cells/ml)
0
2
4
6
8
TIME (days)

that only at an inoculation concentration greater than 1.7×10^5 cells/ml, which corresponds to 7 cells/bead, will the growth be improved by the use of large microcarriers. However, the performance with larger microcarriers at the inoculum concentration of 1×10^5 cells/ml was better than the theoretical prediction. This is probably due to the deviation from the assumption that the critical cell number is proportional to the surface area or to the diameter raised to the second power. If the critical number were taken to be constant for all microcarriers, or proportional to the diameter but raised to a power less than two, then theoretical prediction would more closely approximate the expermental results.

These findings show that knowing the critical number is useful in determining how to increase the multiplication ratio. Under the conditions outlined above, if the average cell number per bead is larger than the critical number, it is possible to improve cell growth by properly selecting bead size.

1.2.2 REDUCTION OF THE CRITICAL CELL NUMBER

The results presented in section 1.2.1 showed that by properly selecting the microcarrier diameter, the inoculum cell concentration necessary for the growth of FS-4 cells could be reduced about two-fold. However, in order to further reduce the inoculum cell concentration requirement, it is necessary to

reduce the critical number required for cell growth. This can possibly be achieved by improving nutritional conditions for cell growth. A desirable medium for supporting cell growth at low inoculum concentration would be the one which would permit one isolated cell in a vast area or on a single bead to grow. Two experimental approaches were taken to select a better medium for cell growth at low inoculum concentrations. In the first a study was performed on Petri dishes. The results obtained from Petri dishes were confirmed using microcarriers as a second system. These will be described separately below.

(1) Clonal Growth on Petri Plates

When cells are inoculated at a low density onto Petri dishes, each colony formed is the result of clonal growth originating from a single cell. For many cell types, especially normal diploid cells, the cloning efficiency is low. In other words, when the cells are inoculated at a very low density, only a small fraction of the cells are able to grow and to form colonies. The cloning efficiency can be greatly improved by nutritional factors (McKeehan et al. 1981b). Clonal growth on Petri dishes is often used for nutritional studies in cell culture. The growth extent is evaluated from both the number of colonies formed and the size of the colonies. Thus, the growth extent reflects the combined effects of the percentage of survival and the growth rate. To select the best medium for cell growth at low inoculum, different media were tested for their effectiveness in supporting clonal growth of FS-4 cells. The

Figure 1-17. Clonal Growth of FS-4 Cells on Petri Dishes in Different Media. All media were supplemented with 5% FCS. 600 cells were inoculated to each 6cm Petri dish. The CO_2 concentrations in the air were: 10% for DME, 7% for DME/F12 mixture and 5% for F12. Cells were stained after incubating for fourteen days. (A): DME; (B): F12; (C): DME/F12.


A
B
C

colonies formed with three of the media tested are shown in Figure 1-17. Five percent FCS was used in all cases. DME was the medium used regularly in other parts of this study and served as the control. Medium F-12 was selected because it was developed by Ham (1965) to support better clonal growth of animal cells, especially diploid fibroblasts. Compared to DME, medium F-12 contains more amino acids and vitamins but at lower concentrations (see Table I-1 in Materials and Methods). The results show that at the same serum concentration Ham's F-12 medium or the 50/50 mixture of Ham's F-12 and DME resulted in better growth than DME alone. Also tested but not shown here were media 199 and Waymouth 752/1. The cloning efficiency in these media was similar to that in DME. At 2% serum concentration, the growth extent was reduced greatly, but Ham's F12 and the F12/DME mixture were still better than DME alone (data not shown).

(2) Clonal Growth on Microcarriers

The substrate to which cells adhere can often affect cell growth. The cloning efficiency on tissue culture dishes was reported to be greatly improved after the dishes were coated with polylysine or other positively charged polymers (McKeeham and McKeeham, 1980). The serum requirement was also reduced in such polylysine coated dishes. In view of the effect of surface properties on cell growth, it was necessary to test the results obtained from Petri dishes in microcarrier culture. To test the effectiveness of different media in supporting cell growth at low

densities, the principle of clonal growth was applied to microcarrier culture. The results presented in section 1.1.3 showed that the distribution of cells on microcarriers can be predicted by the Poisson distribution. When the mean cell number per microcarrier is less than one, the fraction of microcarriers which acquire more than one cell is small. As a result, most growth arises from single cells on individual microcarriers; thus, almost all cell growth is the result of clonal growth. Twelve days after inoculation a significant difference in the extent of cell growth in different media could be seen. A large fraction of the microcarriers in the culture with the DME/F-12 mixture were confluent. With the DME medium, the cells on the microcarriers were sparse and the fraction of microcarriers on which significant growth had occurred was also smaller. The net result was a slower growth rate and lower viability. The extents of cell growth from these three separate experiments are shown in Table 1-1. The DME/F-12 mixture was better than F-12 alone, which in turn was better than DME for growth at low inoculum on microcarriers. The estimated doubling time for cell growth in the DME/F-12 mixture was about 50 to 60 hours, which was close to the typical doubling time of FS-4 cells on microcarriers.

The results also indicated that the growth model developed in this study was simplified to some extent. In the model it was assumed that cells would not multiply if the initial cell number were not larger than the critical number. However, the results here show that, even with only one cell per bead in the inoculum

Table 1-1. Growth of FS-4 Cells at Low Inoculum Concentration

	Inoculum Concentration (x10^{4}cells/ml)	Growth Extent (x10^{4}cells/ml)		
		F-12	DME	DME/F-12
Expt. 1	2.5	28		68
Expt. 2	2.5		16	55
Expt. 3	1.0		< 10	21

The microcarrier concentration was 5 g/l. 5% FCS was supplemented to all media. The carbon dioxide concentrations in the atmosphere of the humidified incubator were 10% for DME, 7.5% for the DME/F-12 mixture and 5% for F-12.

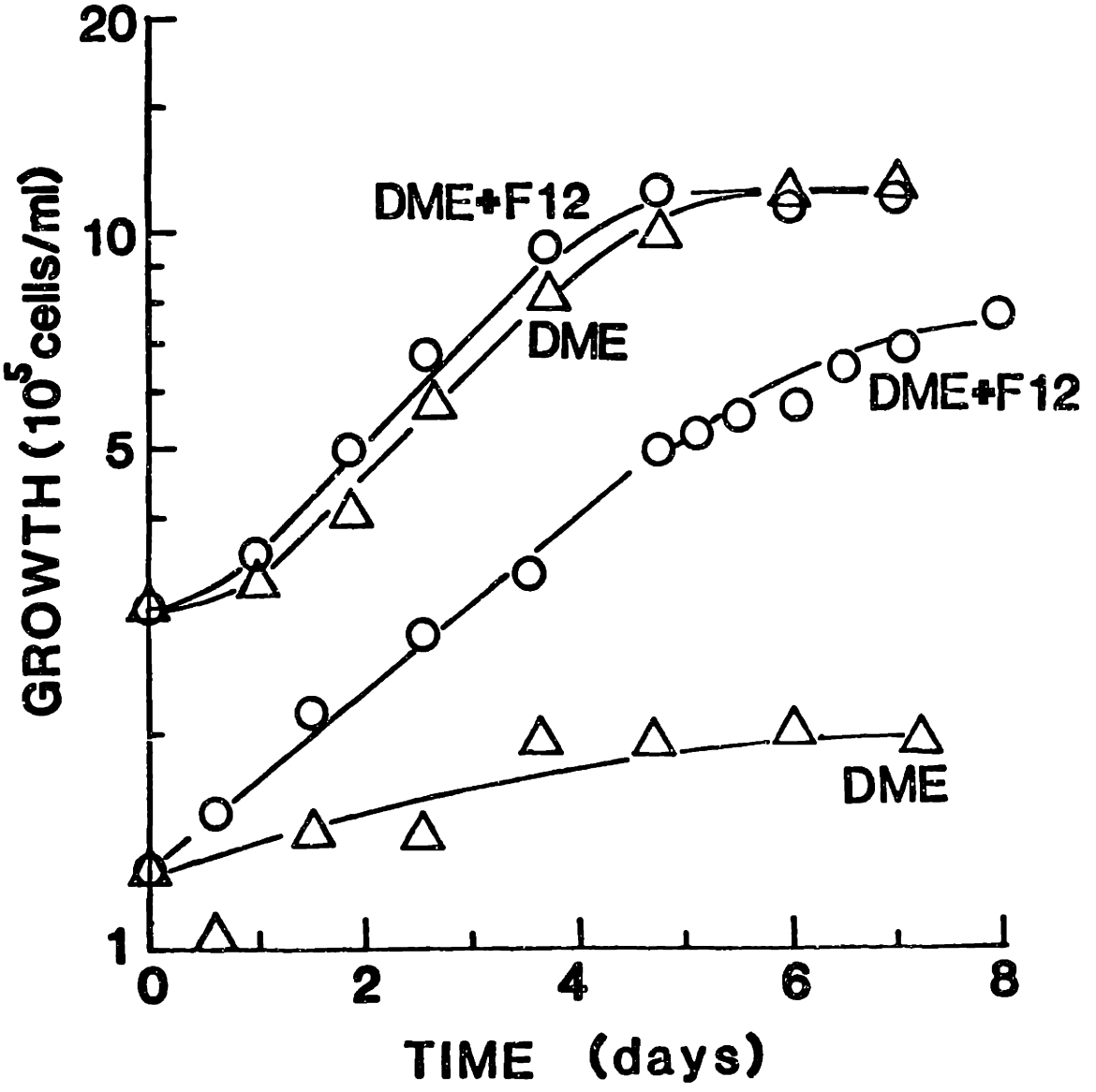
and a critical number of six, some fraction of cells do grow in medium DME, but to a lesser extent.

This technique of clonal growth on microcarriers may be useful in the development of a serum-free medium for microcarrier cultures. To support serum-free growth, a battery of growth factors have to be tested and their optimal concentrations defined. A rapid and facile screening method is necessary for this purpose. Clonal growth on microcarriers can be studied in microtiter plates, in which both the cloning efficiency and the growth rate can be evaluated easily by microscopic examination.

To test the effectiveness of the DME/F-12 mixture in the reduction of the inoculum concentration, growth kinetics in DME/F-12 mixture were compared to those in DME alone. With a high inoculum concentration (3×10^5 cells/ml), cells grew normally to confluence in both media. When the inoculum was reduced to 1.3×10^5 cells per ml, the growth was improved by employing the DME/F-12 mixture. The growth rate in this improved medium was similar to that observed in culture with a high inoculum. However, the growth rate began to decrease at a lower state of confluency and the final growth extent was lower than the normal confluent cell density observed in cultures with high inoculum concentrations (Figure 1-18).

The gradual reduction in the growth rate for the low inoculum culture was probably a reflection of an inferior cell distribution on the microcarriers. During inoculation cells attach randomly to the microcarriers. The cells on those microcarriers which aquire more cells will reach confluence and

Figure 1-18. Kinetics of Cell Growth in DME and a DME/F-12 Mixture. The media were supplemented with 5% FCS. The microcarrier concentration was 5 g/l.


20
10
5
1
GROWTH (10^5cells/ml)
DME+F12
DME
DME+F12
DME
0
2
4
6
8
TIME (days)

stop growing while the cells on others are still multiplying. Therefore the observed growth rate will gradually decrease. When the average cell number per bead at inoculation was reduced in the experiment above, the cell distribution was also changed. To ensure a long exponential growth phase, a proper cell distribution on microcarriers must occur along with any reduction of inoculum concentration.

1.2.3 EFFECT OF MICROCARRIER DIAMETER ON THE MULTIPLICATION RATIO

As shown previously, cell attachment to microcarriers can be modelled using the Poisson distribution function. The shape of the distribution is effected by the mean cell number per bead, which in turn depends on the diameters of the microcarriers. Thus, microcarrier diameter can have a profound effect on cell growth at low inoculum concentrations. A Poisson distribution approaches a normal distribution when the mean is large. Therefore the effects of microcarrier diameter on growth in microcarrier culture will be demonstrated using a normal distribution. When the Poisson distribution approaches a normal distribution, the standard deviation, σ, can be approximated by the square root of the mean, $U^{1/2}$. In a normal distribution, 68% of the population falls within one standard deviation of the mean. Therefore, the upper bound of this 68% of the population

can be approximated as $U + U^{1/2}$ and the lower bound as $U - U^{1/2}$. The ratio of the cell number per microcarrier at the upper bound to that at the lower bound reflects the distribution of cells on microcarriers. The smaller the ratio, the shorter the time lag for microcarriers having the cell number of the lower bound to reach confluence after those at the upper bound have reached confluence. This ratio, $(U + U^{1/2})/(U-U^{1/2})$, is obviously a function of the mean cell number per microcarrier, U. The first derivative of this ratio with respect to the mean is shown in Equation 19:

$$\frac{d}{dU}\left(\frac{U+U^{1/2}}{U-U^{1/2}}\right) = \frac{-U^{1/2}}{(U-U^{1/2})^2}$$

(Eq. 19)

Equation 19 will give derivetives with negative values for all positive values of the mean. This behavior shows that the ratio of the upper bound to the lower bound is a decreasing function of the mean. Thus, increasing the mean cell number per bead should improve the cell distribution, allowing a more uniform exponential growth period. Furthermore, cell distribution on microcarriers can also be improved by increasing the microcarrier diameter.

Using Equations 11 and 12, the growth kinetics on microcarriers of different diameters can be simulated. It is assumed in the following analysis that any single cell on any individual microcarrier is able to grow normally to confluence.

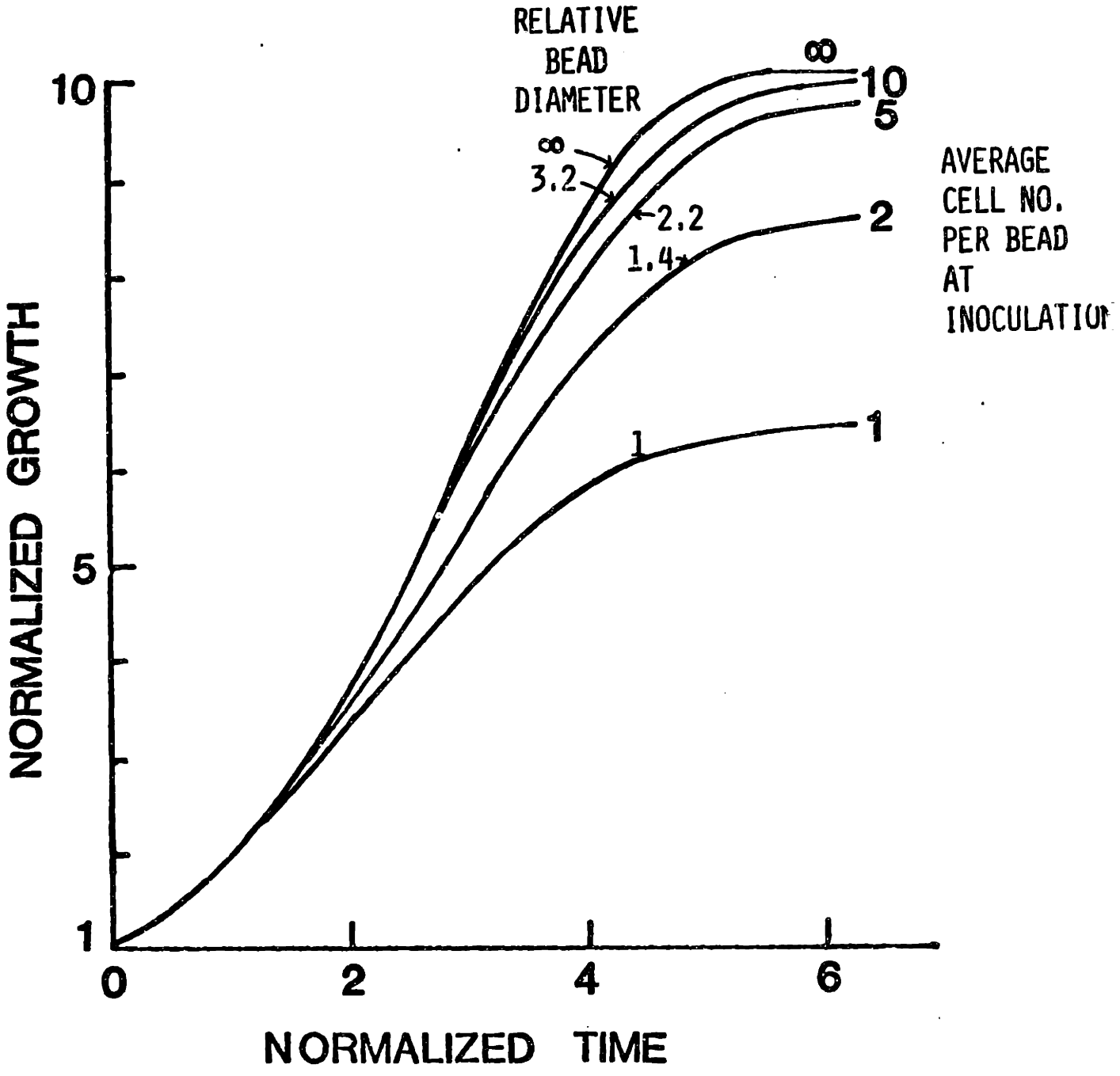
In this simulation, the concentrations of microcarriers of different diameters will be varied in such a way as to maintain equal surface area per unit volume. A ten fold increase in cell concentration, or a multiplication ratio of ten, is the target of this simulation. The results are shown in Figure 1-19. The relative diameter and the average cell number per microcarrier at inoculation for the cases analyzed are shown in Figure 1-20 along with the simulated growth kinetics. For instance, the smallest microcarriers in this simulation have a relative diameter of one. Their confluent cell density is ten cells per microcarrier. An inoculum cell density of one cell per bead on the average is used to allow for a ten fold increase in cell number for all cases of cells on microcarriers growing to confluence. The simulated growth kinetics demonstrate that the microcarrier diameter has a significant effect on both the growth extent and the duration of exponential growth. A proper selection of the microcarrier diameter is needed to achieve a desired multiplication ratio at low inoculum concentration.

For FS-4 cells, a ten- to fifteen-fold increase in cell number can be achieved on Petri dishes. To achieve a multiplication ratio of fifteen, the microcarrier diameter should be increased by about 40% above the size normally used. This corresponds to an initial cell number per bead is about eight to ten on the average.

The effect of microcarrier diameter on the multiplication ratio was then tested experimentally. The results are shown on Figure 1-20. The growth kinetics of FS-4 cells on microcarriers

Figure 1-19. Simulation of Cell Growth on Microcarriers of Different Diameters. The total growth surface area per unit volume is kept constant. The average cell number per bead at inoculation and the relative bead diameters are shown. The saturation cell density for each particular bead diameter is ten times the inoculum average cell density.

EFFECT OF BEAD DIAMETER ON CELL GROWTH
THEORETICAL ANALYSIS

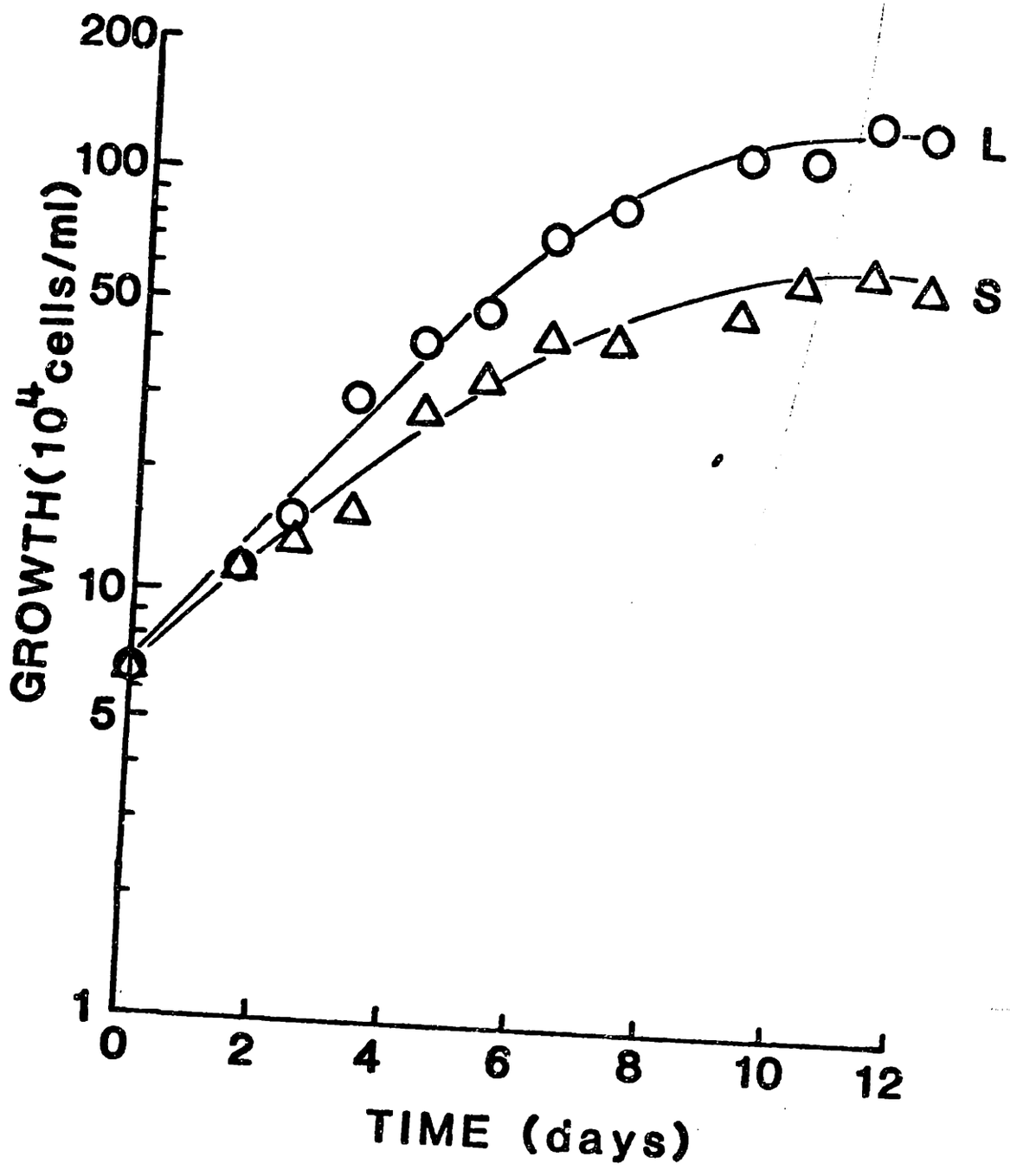


of two different median diameters were compared. The larger microcarriers had a median diameter of 265 μ the smaller microcarriers, 185 μ as routinely used in this thesis. The medium DME/F-12 mixture was used to ensure a low critical cell number for growth. At the two inoculum concentrations tested, confluent growth was achieved only with the microcarriers having a median diameter of 265 μ. The regular 185 μ diameter microcarriers and the low inoculum concentration used resulted in a shorter exponential growth stage as well as a lower growth extent. For FS-4 cells, a typical multiplication ratio in a single stage culture using regular microcarriers is less than four fold. With the combination of improved medium and larger microcarriers, a sixteeen fold increase in cell number can be achieved.

In this analysis, the effect of surface curvature on cell growth has been neglected. It has been reported that on glass fibers of diameters less than 100μ, chick embryo fibroblastic cells preferentially spread out along the longitudinal direction (Fisher and Tickle, 1981). It is thus possible that cells on extremely small microcarriers do not behave in the same ways as those on larger microcarriers. In some rare cases , however, very small microcarriers which had escaped during the size screening process were seen in the bead preparations and microscopic examination revealed no differences in cell growth on these small beads compared to other normal-sized beads.

To achieve an efficient process for mammalian cell cultivation on microcarriers, it is desirable to increase the

Figure 1-20. Effect of Microcarrier Diameter on Cell Growth at Low Inoculum. The surface area per unit volume was the same for cultures with microcarriers of both diameters. (○): microcarriers with median diameter 265 μ at 7g/l; (△): microcarriers with median diameter 185 μ at 5g/l. The medium used was a 50/50 DME/F-12 mixture with 5% FCS.


200
100
50
10
5
1
GROWTH(10^4cells/ml)
L
S
0
2
4
6
8
10
12
TIME (days)

multiplication ratio attainable in batch culture. In this chapter a mechanistic analysis of the inoculum requirement has been presented. To analyse the inoculum requirement a critical cell number for cell growth was identified. The reduction of this critical number was achieved by the use of a better medium for clonal growth. A combination of the better medium and larger microcarriers allowed the achievement of a fifteen fold increase of the cell number in batch culture.

CHAPTER 2. SERIAL PROPAGATION OF ANIMAL CELLS ON MICROCARRIERS

2.1 STRATEGIES OF SERIAL PROPAGATION ON MICROCARRIERS

Anchorage-dependent mammalian cell culture is typically performed in a batch mode where cells grow on a compatible surface to confluence. The confluent cells are then detached and reinoculated onto a larger surface area for further multiplication. However, a suitable method of detaching cells from microcarriers has not been developed and thus roller bottles are necessary for the initiation of a microcarrier culture. This problem in the past has been compounded by the inability to achieve a high multiplication ratio in a batch culture. The large number of roller bottles required for the inoculation of a single microcarrier culture makes inoculum acquisition a prohibitive task, especially in large scale operations. This problem can be alleviated in part, by increasing the multiplication ratio during batch culture. The results presented in Chapter 1 demonstrated that the inoculum cell concentration could be reduced about five-fold by the selection of the proper medium and microcarrier diameter. However, even with an improved multiplication ratio, the number of roller bottles required for the inoculation of a large scale microcarrier culture is staggering. A method of direct inoculation from a seed microcarrier culture is essential for large scale operations.

In order to achieve serial propagation of cells in a microcarrier culture system, one must be able to transfer cells from cell-covered microcarriers to new, bare microcarriers. The results presented in Section 1 showed that the cell distribution on microcarriers after inoculation is critical for subsequent growth. A second important consideration is to develop a direct inoculation method which will ensure a suitable cell distribution after subculturing from a seed microcarrier culture. Furthermore, a reasonable multiplication ratio should be maintained during serial propagation in order to allow large-scale operations with a minimal number of propagation steps.

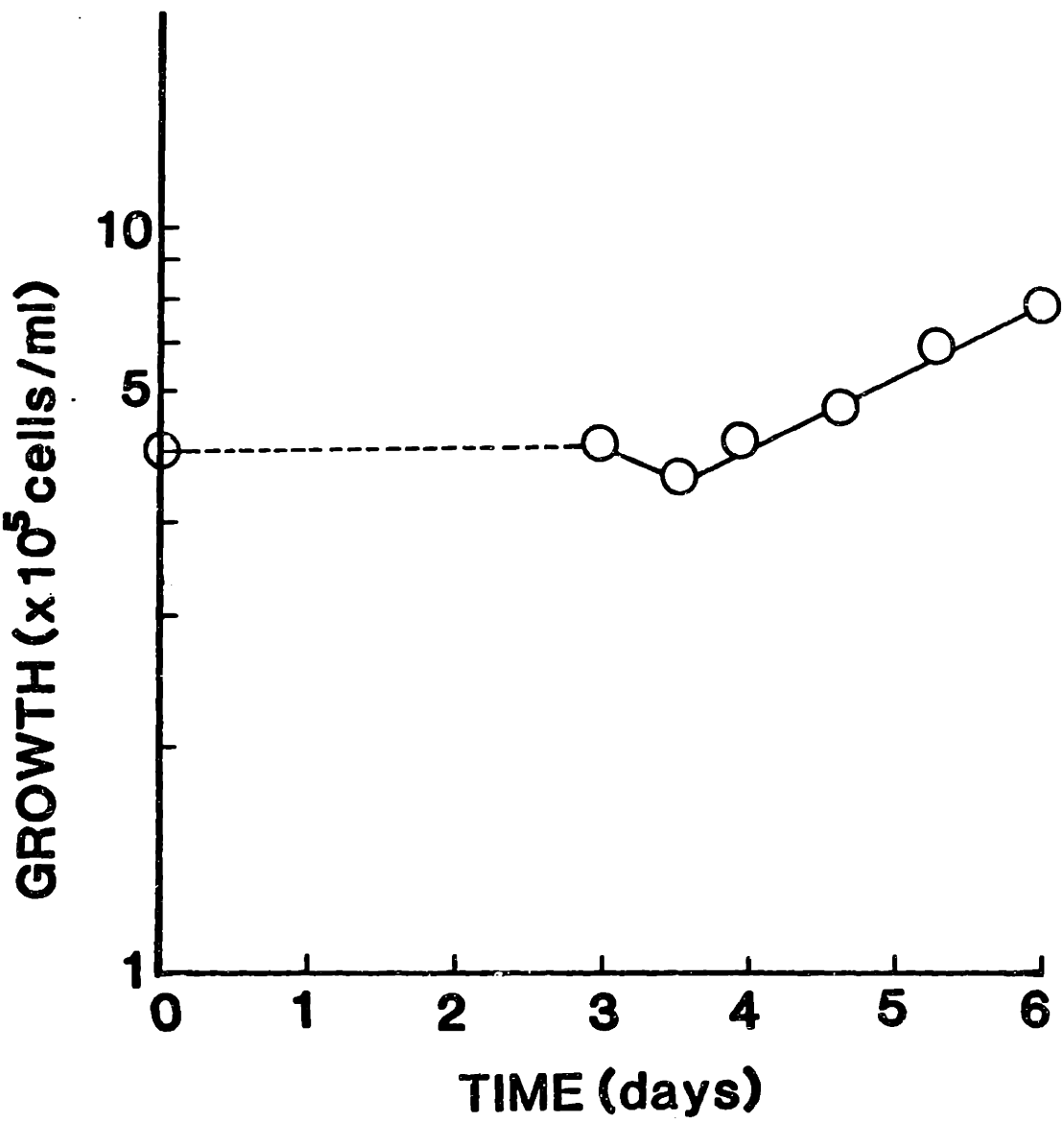
At least two approaches can be taken to inoculate cells directly from a seed microcarrier culture. One is to remove cells from old, confluent microcarriers and then to allow them to reattach onto and grow on new, bare microcarriers. Alternatively, for those cell types capable of movement, such as FS-4 cells and other fibroblasts, direct inoculation could possibly be achieved by cell migration from old microcarriers to new microcarriers. Both of these approaches were tested. The results of the second approach will be presented first.

2.2 THE USE OF A PACKED-BED REACTOR FOR DIRECT INOCULATION

Cells can migrate from one microcarrier to another only when the two microcarriers are in contact. Cell migration is a relatively slow process; typical fibroblasts migrate at a rate of only a few micrometers per hour. An elongated fibroblastic cell can stretch to 100 to 200 micrometers in length. Thus, for cells to migrate from confluent microcarriers to new bare ones, it is necessary for the new and old microcarriers to be in contact for a considerable length of time. During this cell migration period, adequate nutrient supply and removal of metabolites are necessary to sustain cell metabolism and viability.

To allow cells to migrate from confluent microcarriers to newly inoculated bare ones, the use of a packed bed was considered. A preliminary experiment was performed with microcarriers settled on Petri dishes thus making microscopic examination possible in the packed-bed culture. Cells were grown to confluence at a microcarrier concentration of 5 g/l. For each Petri dish, 50 ml of the confluent culture were added and allowed to settle. The settled cake consisted of 6×10^7 cells on 250 mg microcarriers and the cake volume was about 5.5 ml. To the confluent microcarriers, 250 mg of new, bare microcarriers were added. The resulting microcarrier mixture was resuspended in 20 ml of DME supplemented with 5% FCS and placed in a 10 cm bacteriological Petri dish. The packed bed height was about 0.2 cm. Petri dishes were incubated at 37 °C. The medium was changed

Figure 2-1. Direct Inoculation using a Stationary Culture. Confluent microcarriers were mixed with an equal amount of new microcarriers and incubated in Petri dishes with daily medium changes. On the third day the microcarriers were resuspended and transferred to a 250 ml vessel and incubated under normal conditions. Data are normalized to 5 g/l of microcarriers. Stationary stage is shown with broken line.

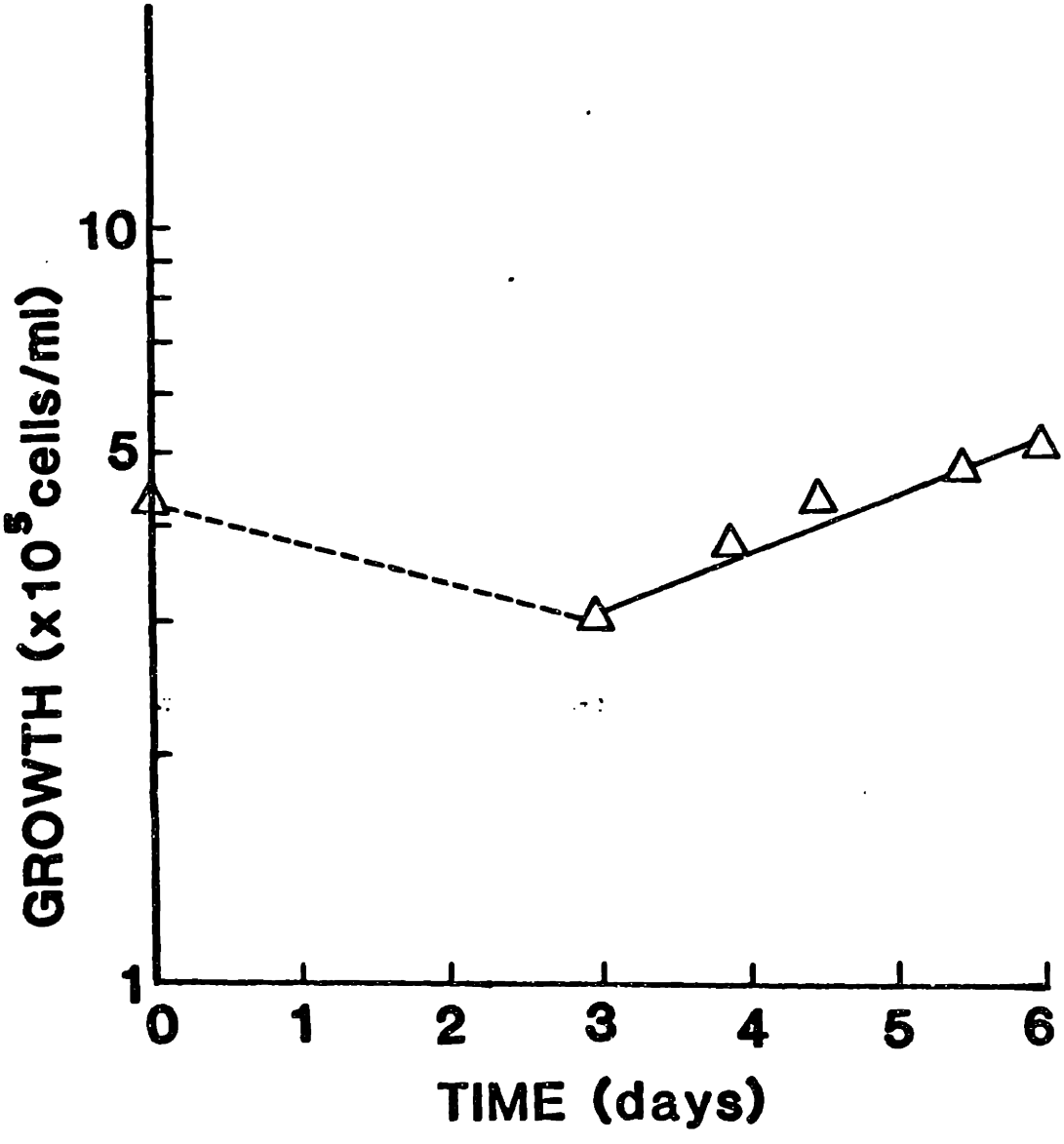

GROWTH (x10^{5}cells/ml)
10
5
1
0
1
2
3
4
5
6
TIME (days)

carefully each day so as to avoid disturbance of the bed. Microscopic examination on the third day showed that most of the microcarriers had cells on them and that only a very small fraction of the old microcarriers were still confluent. The microcarriers from each Petri dish were then resuspended in 100 ml of medium and cultivated in a 250 ml vessel. The results are shown in Figure 2-1. Total cell number did not increase in the packed-bed stage. After the static incubation, many cells were bridged between microcarriers, resulting in clumping of microcarriers. Nevertheless, growth did occur after the microcarriers were resuspended.

A prototype packed bed culture using a chromatographic column was then tested. A chromatographic column (1.5cm in diameter, 10 cm in height) was used instead of a Petri dish. One hundred ml of medium were circulated from a reservior to the column at a flow rate of 15 ml/hr. However, fluid chanelling became a serious problem in this packed column. A significant decrease in cell number was observed in the packed bed. Nevertheless, cell migration onto new, empty microcarriers was observed. The cell number increased subsequently after the microcarriers were resuspended in a spinner vessel (Figure 2-2).

The success of a packed-bed reactor for direct inoculation of a microcarrier culture is largely dependent upon the rate at which cells migrate onto new microcarriers. For FS-4 cells, the rate of migration is too slow for this approach to be an effective means of direct inoculation. The coordination number, or the number of the nearest neighboring spheres, for close-

Figure 2-2. Direct Inoculation Using a Packed-bed Microcarrier Culture. Packed-bed stage is shown with a broken line.


GROWTH (x10^{5}cells/ml)
10
5
1
0
1
2
3
4
5
6
TIME (days)

packed spheres of the same diameter is twelve. Because it is essential for bare microcarriers to contact confluent microcarriers, the theoretical maximum multiplication ratio is therefore twelve using this approach. Another difficulty with the packed-bed system resides in its plug flow nature. Severe gradients of nutrient and metabolite concentrations can be expected in a large scale operation. For those cell types with a high oxygen demand, oxygen will become limiting even in a bed only a few inches high. These problems suggest that the direct transfer using a packed bed may not be optimal. Therefore, an alternative approach was considered and is presented below.

2.3 DIRECT INOCULATION FROM MICROCARRIER CULTURE THROUGH CELL DETACHMENT

A prerequisite for the detachment of cells from a surface is the exposure of the cells to dissociating agents. Cells grown on conventional glass or plastic surfaces are typically treated with proteolytic agents, such as trypsin or collagenase. For some cell types, such as SV-40 transformed 3T3 cells, the removal of cells from the tissue culture substrate can be achieved by treatment with the calcium-specific chelating agent EGTA alone without the use of any proteolytic agents. Many other cell types, on the other hand, must be treated with a trypsin solution containing the divalent cation chelating agent EDTA to facilitate

cell detachment. Proteolytic agents are known to be able to dissociate cells from each other, and from cell culture surfaces. Cells thus treated become more spherical in shape. A subsequent mechanical perturbation, such as repeated pipetting or shaking, releases cells from the surface. Since the morphological change to a spherical shape preceeds cell detachment, this was therefore used as a screening criterion for sucessful cell detachment from microcarriers. Many proteolytic agents, such as trypsin, pronase and collagenase, as wll as cytoskeleton disrupting agents such as cytochalasin B were tested. All of them failed to effect morphological changes in the cells at pH 7.3. In a recent review, McKeehan et al. (1981) reported that, for normal diploid human or chick embryo fibroblasts, no suitable substitute for trypsin as the cell dispersing agent had been discovered. Low concentrations of collagenase, hyaluronidase, and elastase did not release cells. Thus, trypsin alone was chosen for subsequent development of the proper conditions for cell detachment in this thesis.

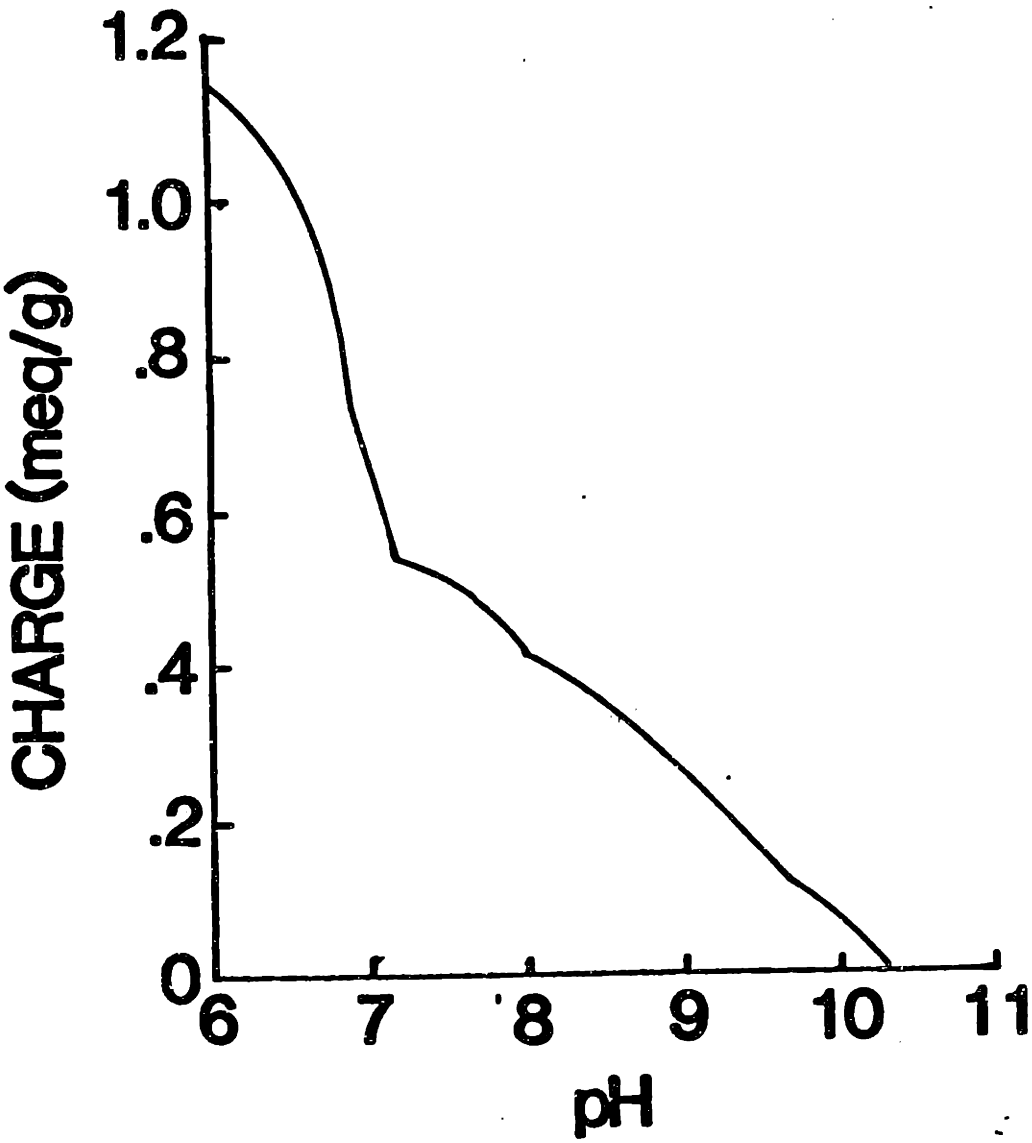
The growth surface of the microcarriers employed in this study is different from the conventional glass or plastic cell culture surfaces in many ways. One of the major differences is the presence of charged DEAE groups on microcarriers. A proper charge density is necessary for optimal cell growth. It is logical to suspect that the charged DEAE groups may play an important role in impeding cell morphological changes and detachment during trypsinization. If charge density does impede

cell dissociation from microcarriers, then reducing the charge density during trypsinization should facilitate cell detachment.

The affinity between DEAE and charged molecules that bind to Sephadex microcarriers can be altered by varying salt concentration or pH. These two methods are often used in eluting bound proteins in ion exchange chromatography. In the screening of a possible cell detachment method, a trypsin solution either at pH 9 or in 1 M NaCl was found to facilitate cell morphological changes indicative of detachment. Cells so treated at pH 9 could revert to the normal elongated cell shape after being transferred to normal growth medium. However, cells treated with 1M sodium chloride solution never regained their normal cell shape when returned to normal growth conditions. Thus only the effect of pH was studied further, although varying the salt concentration might also prove to be effective if a proper concentration was identified.

To evaluate the effect of pH on the charge density of microcarriers, one gram of microcarriers was titrated in an isotonic sodium chloride solution as described in Materials and Methods. The charge density of the microcarriers used in this study, as measured by the chloride ion exchange capacity in 0.1N HCl, was 2 meq/g dextran. The charge density decreases as the pH increases (Figure 2-3). At pH 7.3, the pH of a normal culture used in this study, the effective charge density was 0.5 meq/g dextran. The titration curve shows that at pH 9 the charge density on microcarriers was about 50% of that at pH 7.3. Although the pK_a of DEAE is about 9.5, DEAE-Sephadex showed

Figure 2-3. pH Titration Curve of DEAE-Sephadex Microcarriers.


CHARGE (meq/g)
1.2
1.0
.8
.6
.4
.2
0
6
7
8
9
10
11
pH

multiple pK_a values on the titration curve instead of a single reflection point at pH 9.5. This phenomenon has also been reported by the manufacturer of DEAE-Sephadex, Phamarcia Co.(Phamarcia, technical bulletin) and is attributed to side reactions which occur during the coupling of DEAE to Sephadex beads.

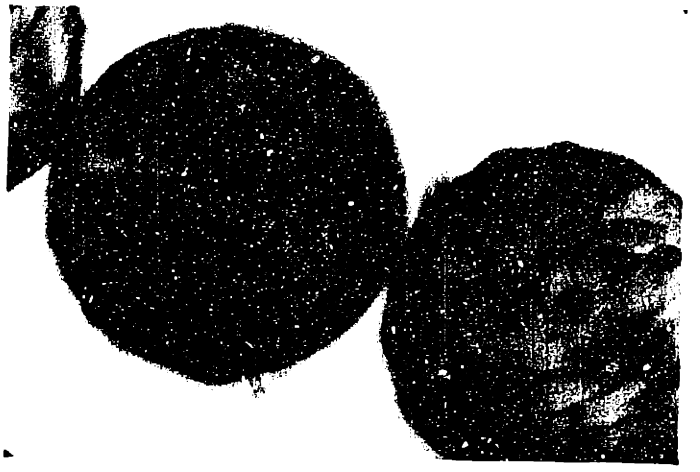
To evaluate the effect of pH, FS-4 cells grown to confluence on microcarriers were washed extensively and resuspended in a 0.2% trypsin solution in 20 mM HEPES buffered at various pH values. After an exposure time of three minutes, a profound effect of pH on cell retraction could be seen (Figure 2-4). With increasing pH of the trypsin solution, cell morphology was observed to change increasingly from an elongated, polarized shape to a more retracted, round form. The retraction of cells from their elongated shape could not be seen at the pH range typically used in cell culture (7.4 - 7.0) even after fifteen minutes of trypsinization.

To determine whether cells trypsinized at a high pH could attach to microcarriers and grow normally, cells grown to confluence on roller bottles were trypsinized at pH 9 and subsequently inoculated into microcarrier cultures. They grew normally until reaching confluence (data not shown). Thus, a short exposure to a high pH did not appear to have any observable adverse effect on the growth of FS-4 cells.

FS-4 cells grown on microcarriers were trypsinized at various elevated pH values. Cells thus treated could be detached from microcarriers by mild mechanical manipulation, such as

Figure 2-4. Morphological Change of Cells Grown on Microcarriers During Trypsinization. (A): before trypsinization. (B): after trypsinization at pH 7.4. (C): after trypsinization at pH 8.6.

A



B

C

repeated pipetting. However, at the culture volume used in this study, 100 to 500 ml, it was more convenient to achieve cell detachment by passing the microcarrier suspension through a small conduit or through a column of packed glass beads as described in Materials and Methods. To quantify the effect of pH on cell detachment, confluent cells on microcarriers were trypsinized at different pH values and subsequently passed through the packed glass-bead column. The results are shown in Figure 2-5. Cell detachment improved progressively with increasing pH. Cells thus detached following high pH trypsinization reattached to microcarriers and retained their viability. A pH range of 8.4 to 8.8 was used for most of the experiments. However, with a longer exposure to trypsin solution (fifteen minutes), a pH of 8.2 was also used successfully to trypsinize, detach and recultivate FS-4 cells.

Using this method, FS-4 cells were serially propagated in microcarrier culture. A multiplication ratio of four was used. After cell detachment, a 100 ml culture at 5 g/l microcarrier concentration was used to inoculate a 400 ml culture at the same microcarrier concentration. The 400 ml culture consisted of 1.5 g of new microcarriers and 0.5 g of carried-over used microcarriers, thus resulting in a concentration of 5 g/l of microcarriers (Figure 2-6).

At the end of the exponential growth phase, cells thus propagated were detached by trypsinization at pH 8.6 again and subsequently used to reinoculate a culture.

Figure 2-5. Effect of pH During Trypsinization on Cell Detachment. (○):0.2% trypsin solution; (●): 0.6% trypsin solution.

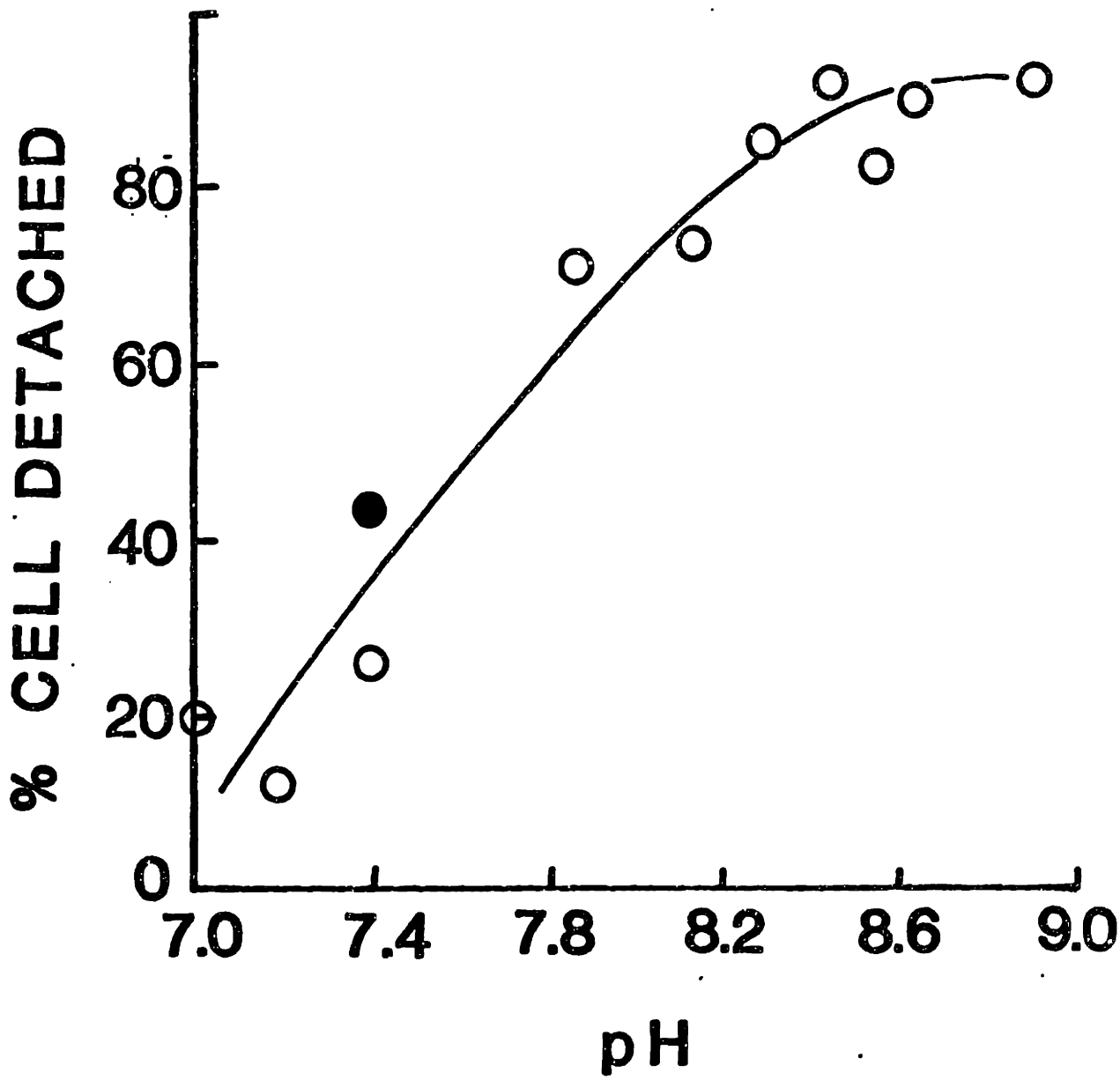
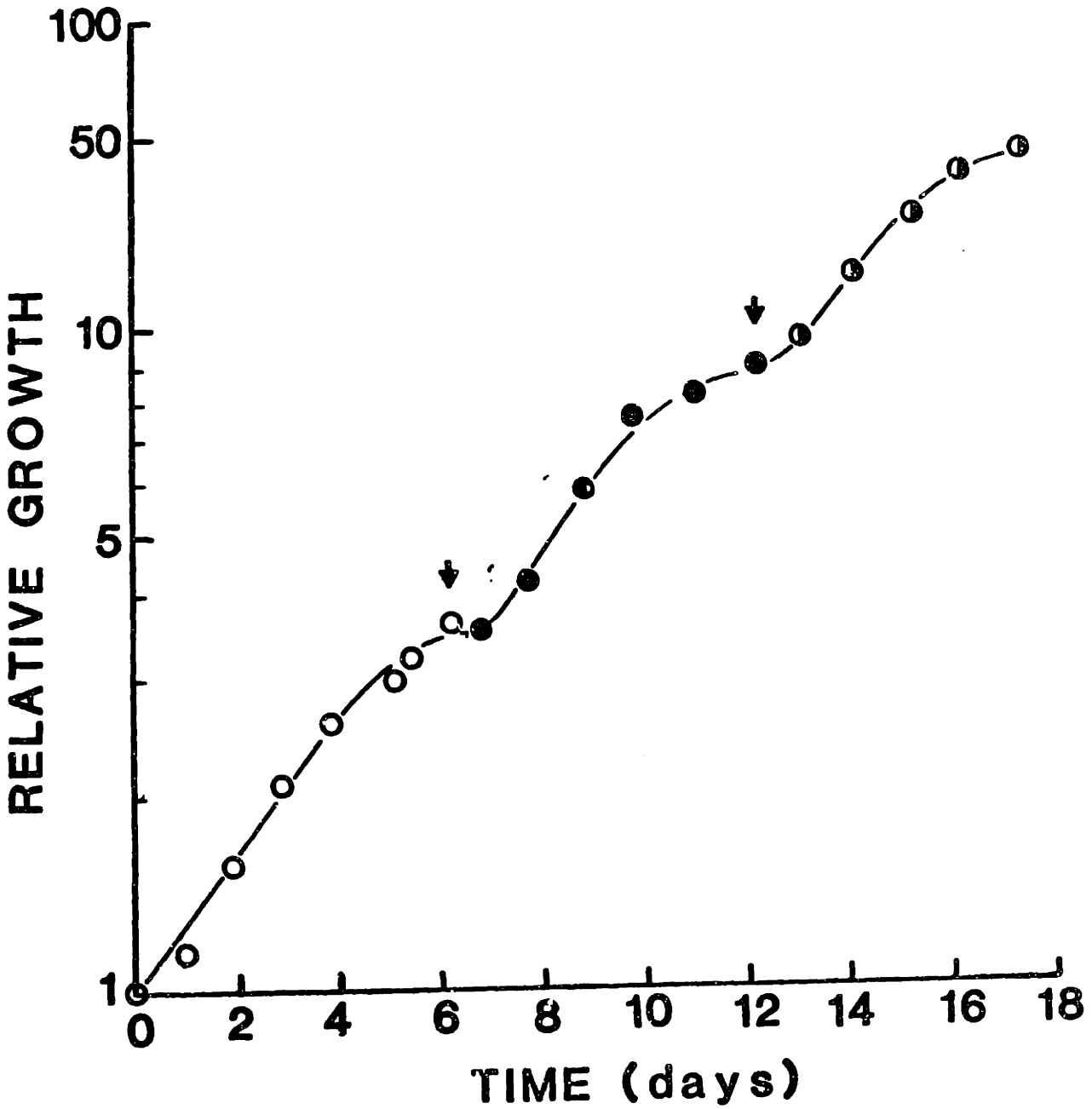
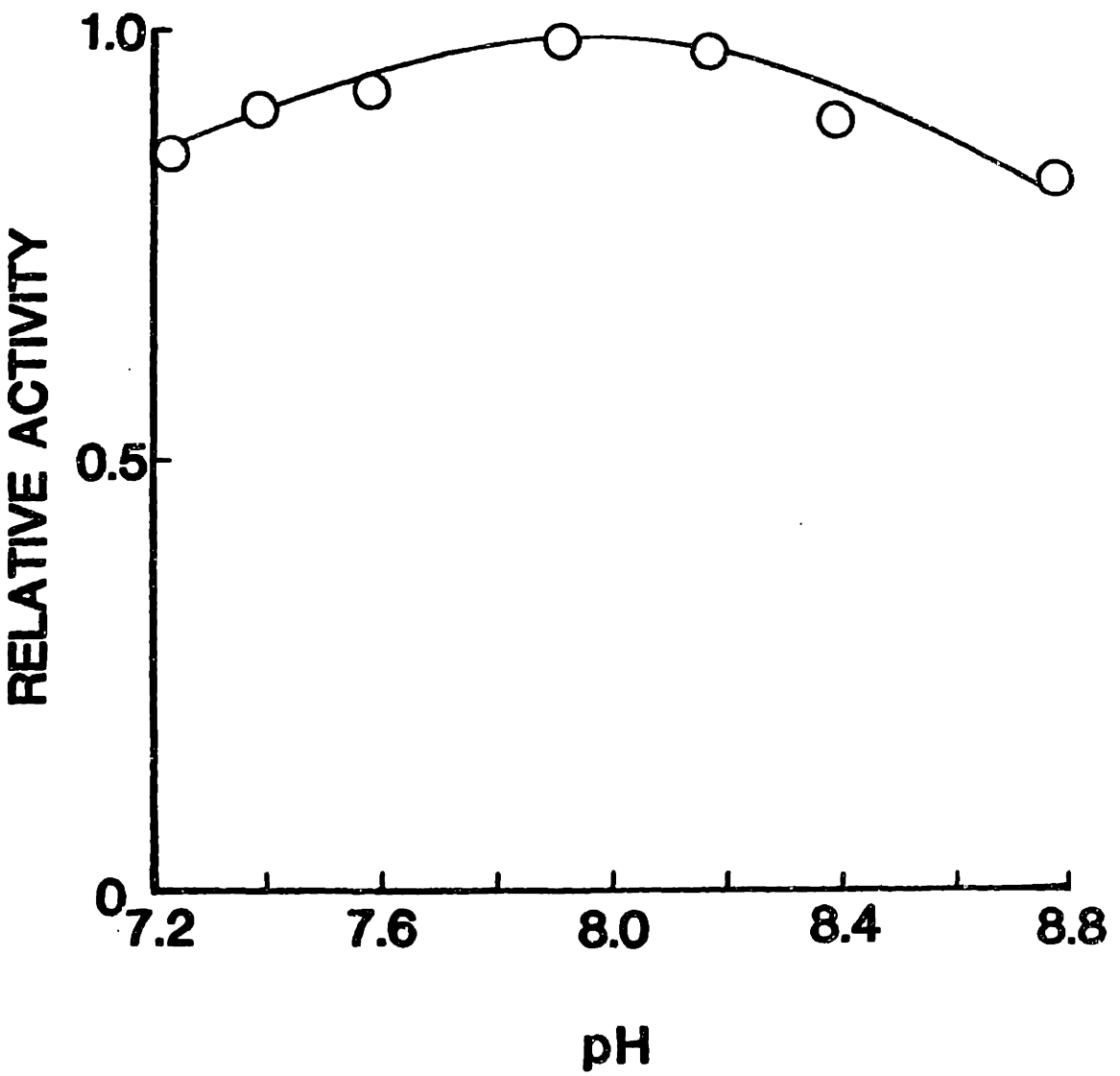

% CELL DETACHED
0
20
40
60
80
7.0
7.4
7.8
8.2
8.6
9.0
pH

Figure 2-6. Serial Propagation of FS-4 Cells on Microcarriers. Arrows show the trypsinization at high pH and reinoculation into a new culture.


100
50
10
5
1
RELATIVE GROWTH
0
2
4
6
8
10
12
14
16
18
TIME (days)

For FS-4 cells, a short exposure to a 0.2% trypsin solution of pH 8.4 - 9.0 resulted in cell morphological changes which allowed cells to be detached from microcarriers and used for subsequent inoculation. Although it was postulated that a charge density effect was responsible for the effect of pH on cell detachment, the true mechanism affecting cell detachment is still not known. The proteolytic activity of trypsin increases as the pH shifts from acidic to more basic range. However, the effect of pH on cell detachment was unlikely to be the result of increased trypsin activity. Increasing the trypsin concentration by three-fold during trypsinization at normal pH failed to induce the morphological changes which preceed cell detachment. Although the number of cells detached was slightly improved, the effect was not as significant as that at high pH's (Figure 2-5). The results shown in Figure 2-5 do not reveal the viability of cells after detachment from microcarriers. Cells detached after morphological changes are observed to reattach to microcarriers with subsequent reinoculation. However, few cells detached at pH 7.4 were able to reattach to either microcarriers or to Petri dishes. Another line of evidence that the improvement in cell detachment was not due to increased proteolytic activity is shown in Figure 2-7. The proteolytic activity of the 0.2% trypsin solution used in this study was assayed at different pH values with a chromogenic substrate (Tomarelli et al. , 1949). The proteolytic activity changes only to a small extent in this pH range. It increases with increasing pH to a maximum at pH 7.9-8.2. Further increase in pH resulted in a decrease in the

Figure 2-7. The Effect of pH on Proteolytic Activity of Trypsin.


1.0
0.5
0
RELATIVE ACTIVITY
7.2
7.6
8.0
8.4
8.8
pH

proteolytic activity (Figure 2-7). Although the initial increase in proteolytic activity coincided with the observed improvement in cell detachment shown in Figure 2-5, the subsequent reduction in proteolytic activity at pH higher than 8.4 was not observed to impede cell detachment. Furthermore, the difference of proteolytic activity in this pH range was too small to account for the effect on cell detachment. This insensitivity of trypsin activity has been reported by Northrop and Kunitz (1932). Using crystalline trypsin, they found that the optimal pH for trypsin was between 7.5 and 9.0 and within this range the activity did not vary with pH. Therefore, the improvement of cell detachment at high pH was due to a mechanism other than an increased proteolytic activity.

In the study of immobilized enzymes, Goldstein et al observed that the net charge of a matrix can create a microenvironment around the solid phase, thus resulting in a change of the characteristics of the enzymes bound to the matrix. The pH activity profile of immobilized enzymes is often different from that of free enzymes. However, the hydrogen ion concentration in the immediate surrounding of a positively charged matrix, such as a DEAE conjugated matrix, is lower than that in the bulk liquid; thus, the observed optimum pH as measured in the bulk liquid should appear to have a lower value than that for the soluble enzyme. This phenomenon of pH displacement toward a more acidic range has been reported in enzymes bound to DEAE-cellulose, including amyloglucosidase (O'Nell et al. , 1971), penicillin amidase (Self et al , 1969)

and α-chymotrypsin (Kay and Lilly, 1970). Therefore, it is very unlikely that the need for higher pH in trypsinizing cells from microcarriers is due to a shift in the optimal pH of trypsin in the microenvironment of DEAE-Sephadex microcarriers.

Well-spread fibroblasts anchor to a substrate through focal contact. The ventral surface of the cell is close to the substrate only in a number of small regions instead of over its whole area (Abercrombie et al. 1971). The points of contact are often called attachment plaques. Those focal attachment plaques are embedded in extracellular matrices (Chen and Singer, 1980). The cell surface components involved in adhesion to a substrate are mostly glycoproteins or proteoglycans. Only a few proteins involved in cell attachment have been identified, such as vinculin and fibronectin. During cell movement and retraction from an elongated cell shape, the cell membrane dissociates from the attachment plaques. The retraction of cells from an elongated shape after trypsinization is an active process which requires cellular energy metabolism (Rees et al., 1977). The process of trypsinization thus involves the cleavage of surface proteins, loosening of the cell-substrate binding, transmission of signals through the membrane to initiate the energy-dependent restructuring of cytoskeleton molecules, and retraction of the cytoplasmic membrane. The processes of cell adhesion, transmembrane signal transmission and retraction are very complex and their mechanisms are still not clear. Although the investigation of charge effect led to successful cell detachment from microcarriers, the molecular mechanism involved in the

nonresponsiveness to trypsin at neutral pH and detachment at high pH is probably much more complicated than a simple charge effect. A report in the early sixties (Mackenzie et al. , 1961) stated that cells retracted from their elongated shape at an elevated pH. However, in that report the cellular retraction was accomplished over several hours, as opposed to a few minutes as observed in this study. Nevertheless, the involvement of a cellular response to high pH in the improvement of cell detachment could not be ruled out.

The cell detachment experiments described in this thesis were all performed at room temperature using a 0.2% trypsin solution containing 0.01% EDTA. In order to maximize cell viability, it is desirable to minimize the trypsin concentration. The trypsin concentration used in this study was chosen because it has been routinely used in this laboratory for cell maintenance in roller bottles. FS-4 cells detached under the experimental conditions reattached to and grew on microcarriers without a significant loss of viability. Thus, no attempts were made to reduce either the trypsin concentration or the temperature of trypsinization. Temperature can often affect cell viability during subculturing. Wang et al. (1968) reported that some cells became more resistant to centrifugal stress when the temperature was lowered. McKeehan (1977) reported that cells trypsinized at a low temperature (4 °C) showed a significant improvement in subsequent clonal growth. For the detachment of FS-4 cells low temperature treatment was not necessary. However, for some other cells which are sensitive to pH variation, a

combination of high pH, low temperature and reduced trypsin concentration may be helpful.

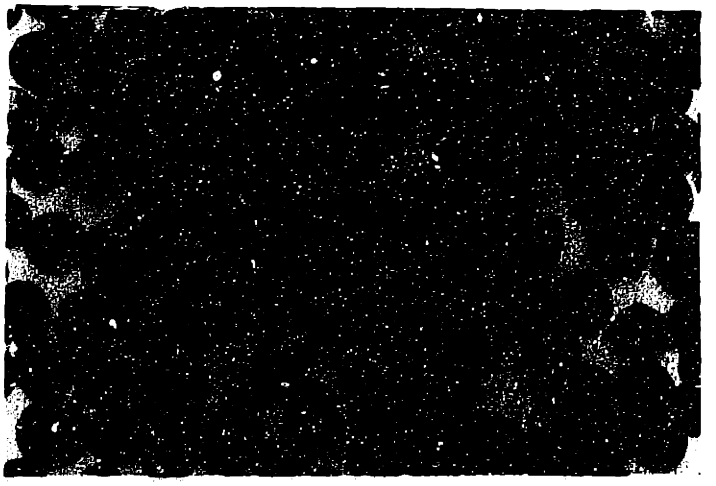
2.4 DIFFFERENTIAL CELL ATTACHMENT TO MICROCARRIERS

By trypsinization at an elevated pH, cells can be detached from microcarriers and reinoculated into a new culture. However, during the subsequent growth after such a bead-to-bead transfer, a nonhomogeneous microcarrier population distribution was observed. Cells grew well on some microcarriers, but poorly on others. An experiment was carried out to determine if this was due to the difference between newly added microcarriers and the old, used microcarriers carried over from the previous culture. Microcarriers of two distinct sizes were used. Cells were first allowed to grow to confluence on the smaller microcarriers. The confluent cells were then trypsinized at pH 9 and inoculated into a new culture consisting of the larger microcarriers. An experiment under the reverse conditions, using cells from the larger microcarriers to inoculate a culture containing new, smaller microcarriers, was also performed, in order to eliminate the effect of microcarrier diameter from the results. At the end of exponential growth, the two cultures were examined microscopically. The results, shown in Figure 2-8, demonstrate that cells grew better in both cases on newly added microcarriers than on microcarriers carried over from the seed culture.

This phenomenon could have been caused by two possible mechanisms. The first possibility is that cells may have attached preferentially to newly added microcarriers after

Figure 2-8. Cell Growth on New and Used Microcarriers after Subculturing. (A): cells grown on small microcarriers inoculated onto large, new microcarriers; (B): cells grown on large microcarriers inoculated onto small, new microcarriers.

A



B

inoculation. The other is that the growth rate on microcarriers on which cells had previously grown may be slower than on new microcarriers. To differentiate between the two possibilities, cells were inoculated into two cultures which were identical except one consisted of new and the other of used microcarriers. The kinetics of cell attachment was followed by measuring the decrease in the concentration of unattached cells. The kinetics were found to be first order with respect to free cell concentration in both cultures, however, the rate of cell attachment to new microcarriers was significantly faster than to used microcarriers (Figure 2-9). After cell attachment both cultures grew at the same rate until confluence (Figure 2-10). The small difference in initial cell concentrations seen in Figure 2-9 is due to the small fraction of cells which remained on the used microcarriers after cell detachment. From the results shown in Figures 2-8 and 2-9, it was concluded that the nonhomogeneous microcarrier population distribution observed in the serial propagation of cells was due to different cell attachment rates to new and used microcarriers, and not to differences in growth rates.

Since the initial cell distribution may have a profound effect on subsequent growth, the differential cell attachment to new and used microcarriers can be a nuisance. To compensate for the inferior cell attachment to used microcarriers, a fraction of the cells could be retained on used microcarriers during cell detachment. To evaluate the feasibility of such an approach the following analysis was performed: An inoculum concentration of

Figure 2-9. Kinetics of Cell Attachment to New and Used Microcarriers. Cells from roller bottles were inoculated into two vessels containing new microcarriers (o), or used microcarriers (Δ) from which cells had been removed by high pH trypsinization. Microcarrier concentration was 5 g/l. The medium used was DME supplemented with 5% FCS.

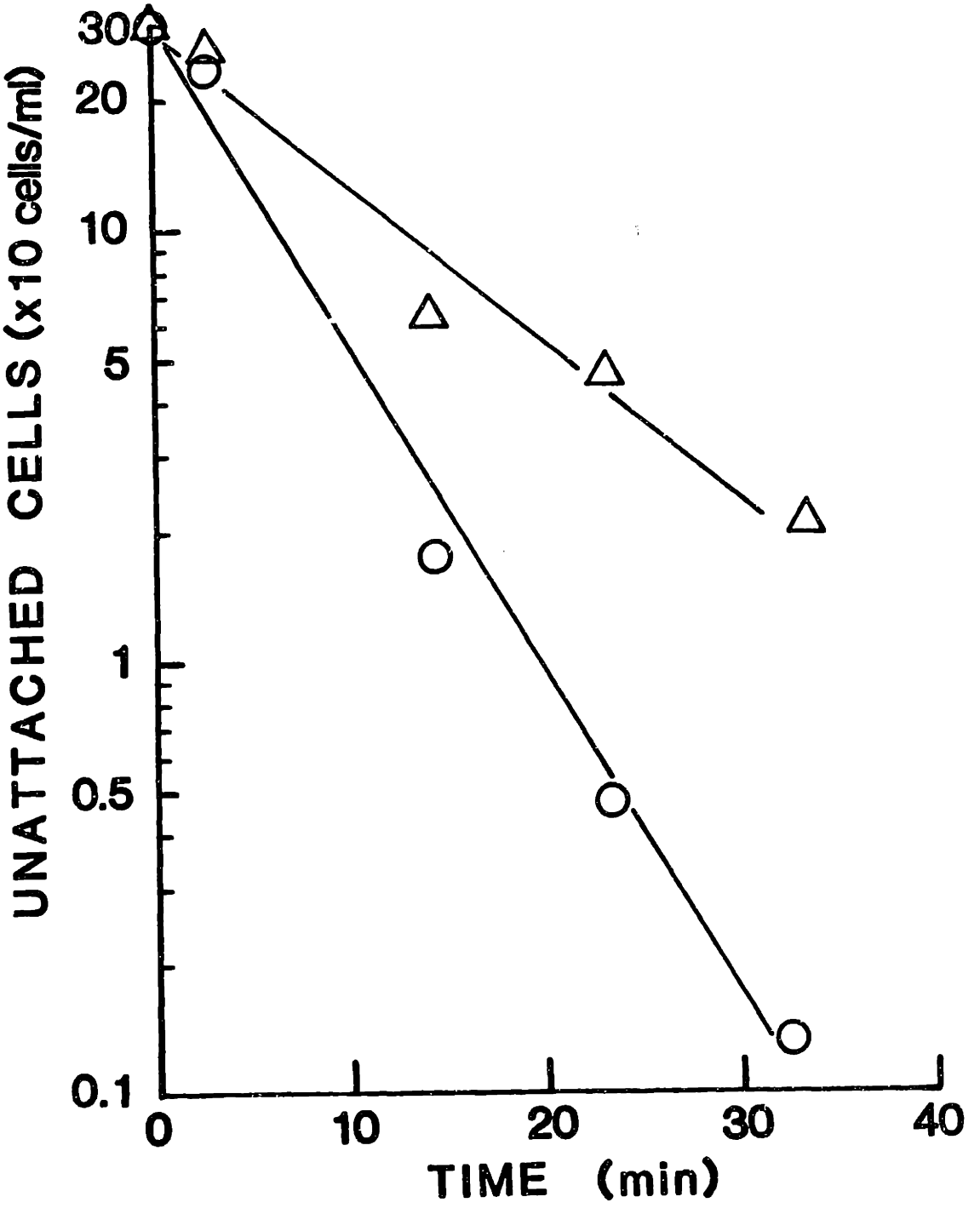
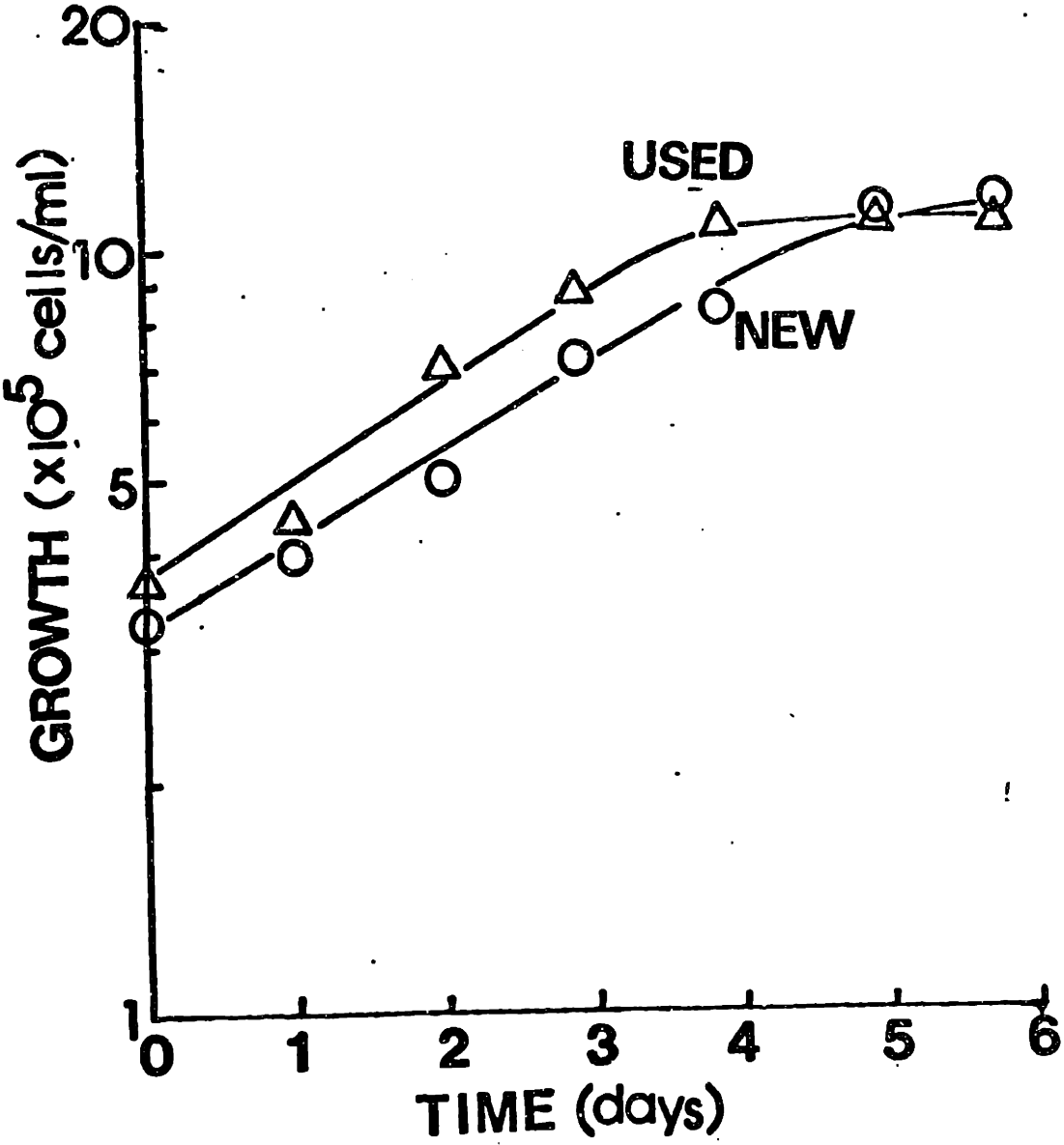

UNATTACHED CELLS (x10 cells/ml)
30
20
10
5
1
0.5
0.1
0
10
20
30
40
TIME (min)

Figure 2-10. Growth of Cells in Cultures of New and Used Microcarriers. (○): new microcarriers; (△): used microcarriers. Experimental conditions were the same as Figure 2-9.


20
10
5
1
GROWTH (x10^5 cells/ml)
USED
NEW
0
1
2
3
4
5
6
TIME (days)

X_o is acquired by detaching cells from the used microcarriers of a seed culture. The new culture to be inoculated contains both new microcarriers and used microcarriers carried over from the seed culture. The fraction of used microcarriers is f, that of new microcarriers is thus 1-f. The achievable multiplication ratio is thus 1/f. Cell attachment to both new and used microcarriers is assumed to be first order with respect to the concentration of unattached cells. The rate constants of cell attachment to the used and new microcarriers are denoted as k_1 and k_2 respectively. The rate of cell attachment to the used microcarriers is expressed in Equation 20.

$$dX_1/dt = k_1(X_o - X_1 - X_2)f$$

(Eq. 20)

That for the new microcarriers is described as Equation 21.

$$dX_2/dt = k_2(X_o - X_1 - X_2)(1- f)$$

(Eq. 21)

In the above equations X_1 and X_2 are the concentrations of cells attached to the used and new microcarriers respectively. At inoculation (t = 0) no cells are on the new microcarriers. However, a number of cells, $X_2(0)$, will be retained on the used microcarriers after cell detachment. Thus, the initial conditions are described by Equations 22 and 23.

(1) $t = 0, \quad X_1 = X_1(0)$

(Eq. 22)

(2) $t = 0, \quad X_2 = 0$

(Eq. 23)

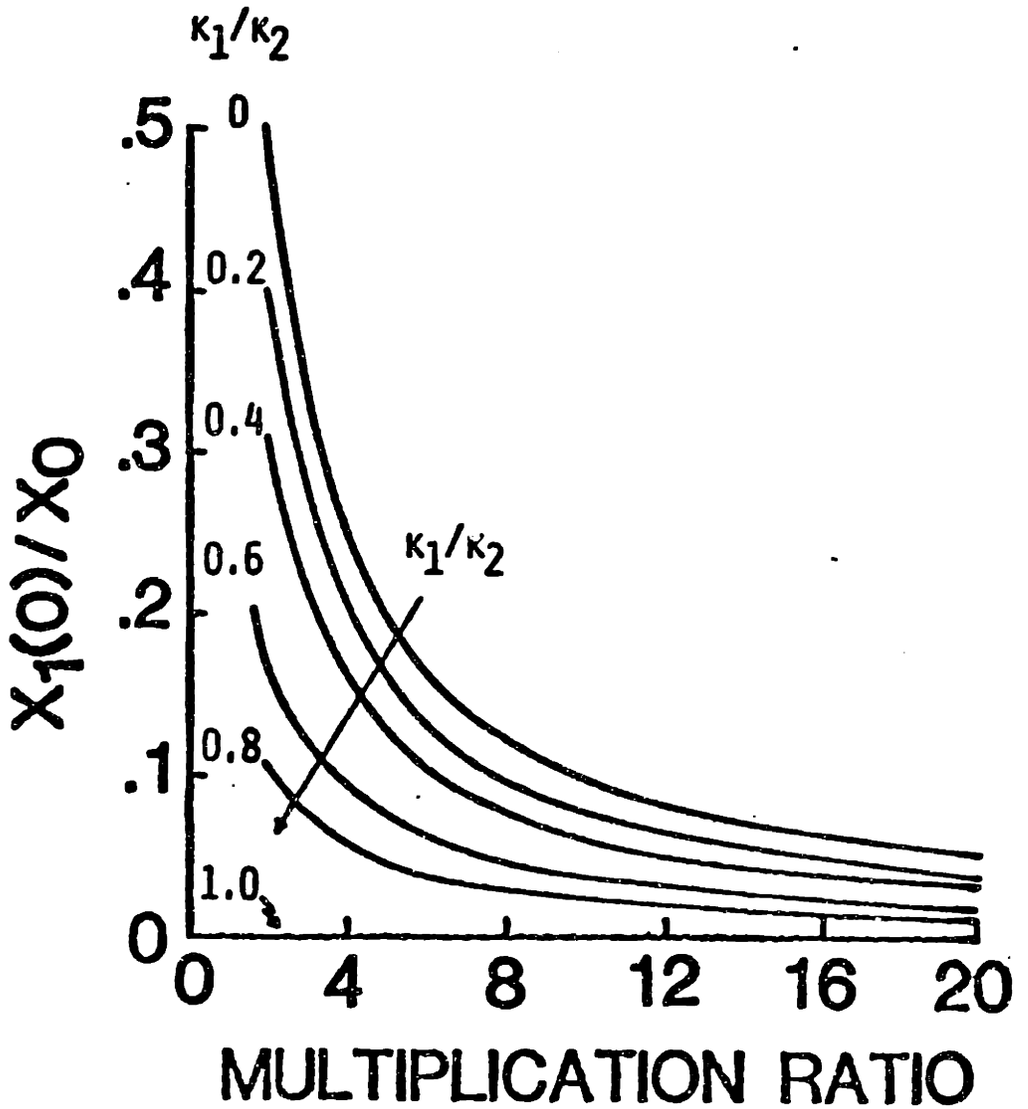
The desired constraint is to have the same average cell number per bead on both new and used microcarriers after cell attachment (t_∞). The ratio of the number of used microcarriers to that of new microcarriers is f/(1-f). Therefore, the constraint can be expressed as Equation 24.

$$X_1(t_\infty)/f = X_2(t_\infty)/(1-f)$$

(Eq. 24)

The simultaneous equations (Eq. 20 and Eq. 21) can be solved analytically. The fraction of the cells which should be retained on the used microcarriers for various multiplication ratios and attachment rate constants is shown in Figure 2-11. Clearly, the fraction of the cells that must be retained on the used microcarriers in order to give rise to an even cell distribution between the new and used microcarriers is greatly dependent on the multiplication ratio and the rate constants of cell attachment.

Figure 2-11. Partial Cell Detachment Required to Achieve Even Cell Distribution after Reinoculation.

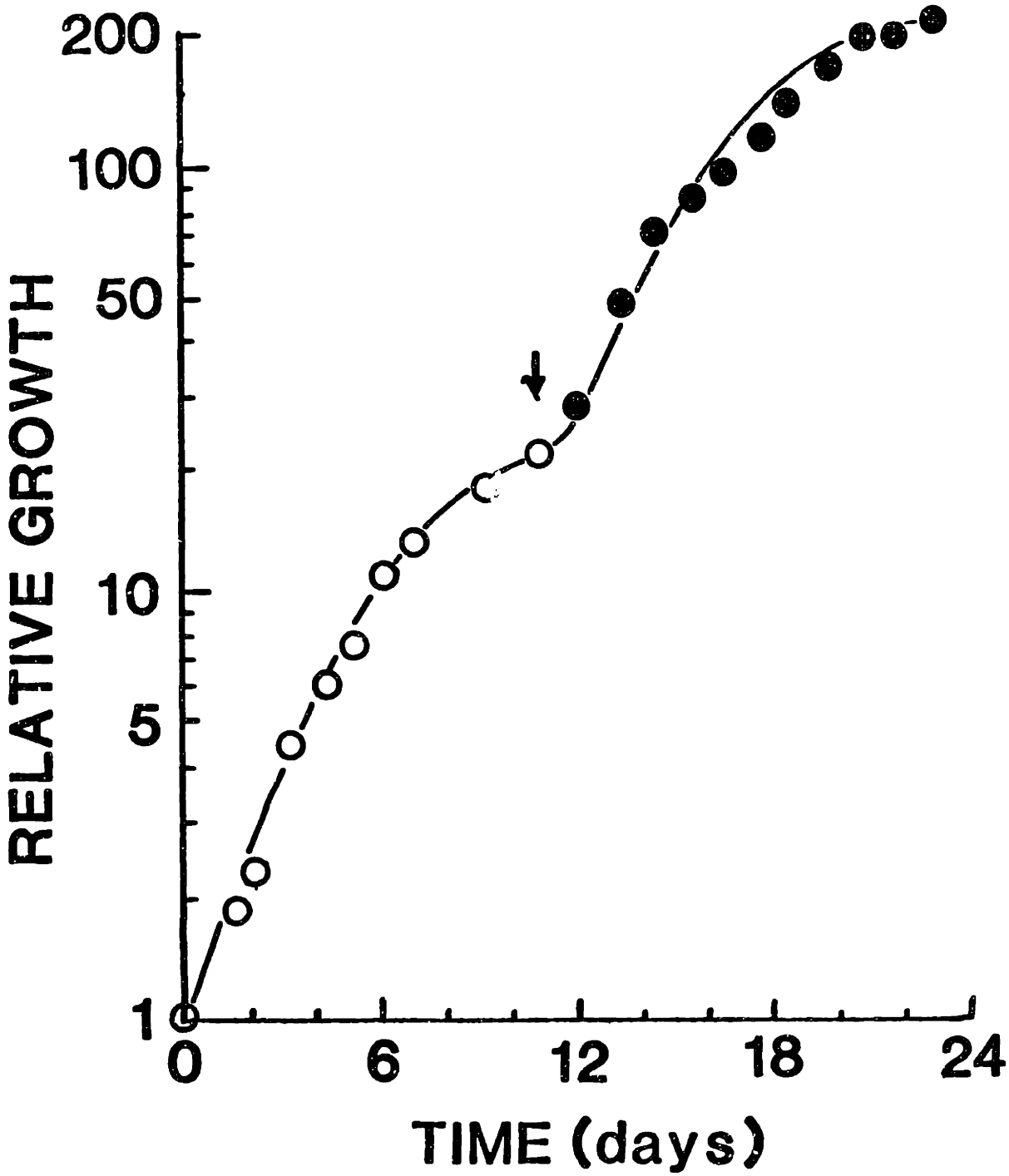

κ_1/κ_2
0
0.2
0.4
0.6
0.8
1.0
κ_1/κ_2
.5
.4
.3
.2
.1
0
$X_1(0)/X_0$
0
4
8
12
16
20
MULTIPLICATION RATIO

2.5 SERIAL PROPAGATION AND PRODUCT FORMATION ON MICROCARRIERS

From the foregoing analysis, it can be seen that, if the multiplication ratio is low, a significant fraction of the cells should be retained on used microcarriers in order to allow an even cell distribution. A precisely controlled partial cell detachment would however be cumbersome and difficult. An alternative to partial cell detachment is to increase the multiplication ratio in order to minimize the effect of differential cell attachment on the cell distribution. This can be achieved by the use of a better medium and a selected microcarrier diameter, as was shown in Chapter 1. An experiment was carried out in which FS-4 cells were inoculated directly from a microcarrier culture at a high multiplication ratio. This is shown in Figure 2-12. Cells were grown on microcarriers having a median diameter 40% larger than the microcarriers routinely used in this study. The DME/F-12 mixture, which was shown to reduce the critical number for cell growth, was used for the cultivation. The confluent cells from the first culture were trypsinized at pH 8.5 and subsequently detached to inoculate the second culture. Cells grew normally to confluence. A total increase in cell number of about two-hundred-fold was achieved in the two stage culture.

To ensure that cells thus propagated are capable of product formation, FS-4 cells serially propagated on microcarriers were induced for β-interferon production using the superinduction

Figure 2-12. Serial Propagation of Cells on Microcarriers of Selected Diameter. The arrow indicates trypsinization and reinoculation. The medium used was a DME/F-12 mixture supplemented with 5% FCS. The microcarrier concentration was 7 g/l. The median diameter was 270 μ.


200
100
50
10
5
1
RELATIVE GROWTH
0
6
12
18
24
TIME (days)

procedure reported by Giard et al. (1981) and described in Materials and Methods. The control culture was inoculated with cells obtained from roller bottles. The production medium was changed one day after the beginning of the production stage. The β-interferon produced in the first- and second-day periods was assayed as described in Materials and Methods. The results are shown in Table 2-1. The interferon productivity of cells serially propagated on microcarriers was comparable to that of those inoculated directly from roller bottles. Thus, FS-4 cells serially propagated on microcarriers at a high multiplication ratio in each batch culture can grow normally and are capable of interferon production.

To test if this subculture procedure is applicable to other cell types, monkey kidney epithelial cells, Vero, were tested. Vero cells were chosen because they are often used for the production of vaccines. Vero cells grown to confluence on microcarriers were subcultured with the same procedure as for FS-4, using a 0.2% trypsin solution at pH 9.0. Vero cells thus cultivated grew very well with no appreciable lag phase or decrease in growth rate (Figure 2-13). After the cells grew to confluence, they were infected with vesicular stomatitis virus (VSV) to test the effect of direct inoculation on virus production. The results are shown in Figure 2-14. The production period lasted for about fifteen hours. The titer of virus obtained was comparable to that reported in the literature (Giard et al., 1977).

Table 2-1. β-INTERFERON PRODUCTION BY FS-4 CELLS GROWN ON MICROCARRIERS

	Interferon titer (units/ml)		
	inoculated from roller bottles	inoculated from microcarriers	
		run 1	run 2
0 -24 hr	24,000	24,000	15,000
24-48 hr	11,000	9.600	7,600

Figure 2-13. Serial Propagation of Vero Cells on Microcarriers. First arrow indicates trypsinization and reinoculation. At the end of cell growth cells were infected with VSV for virus production.

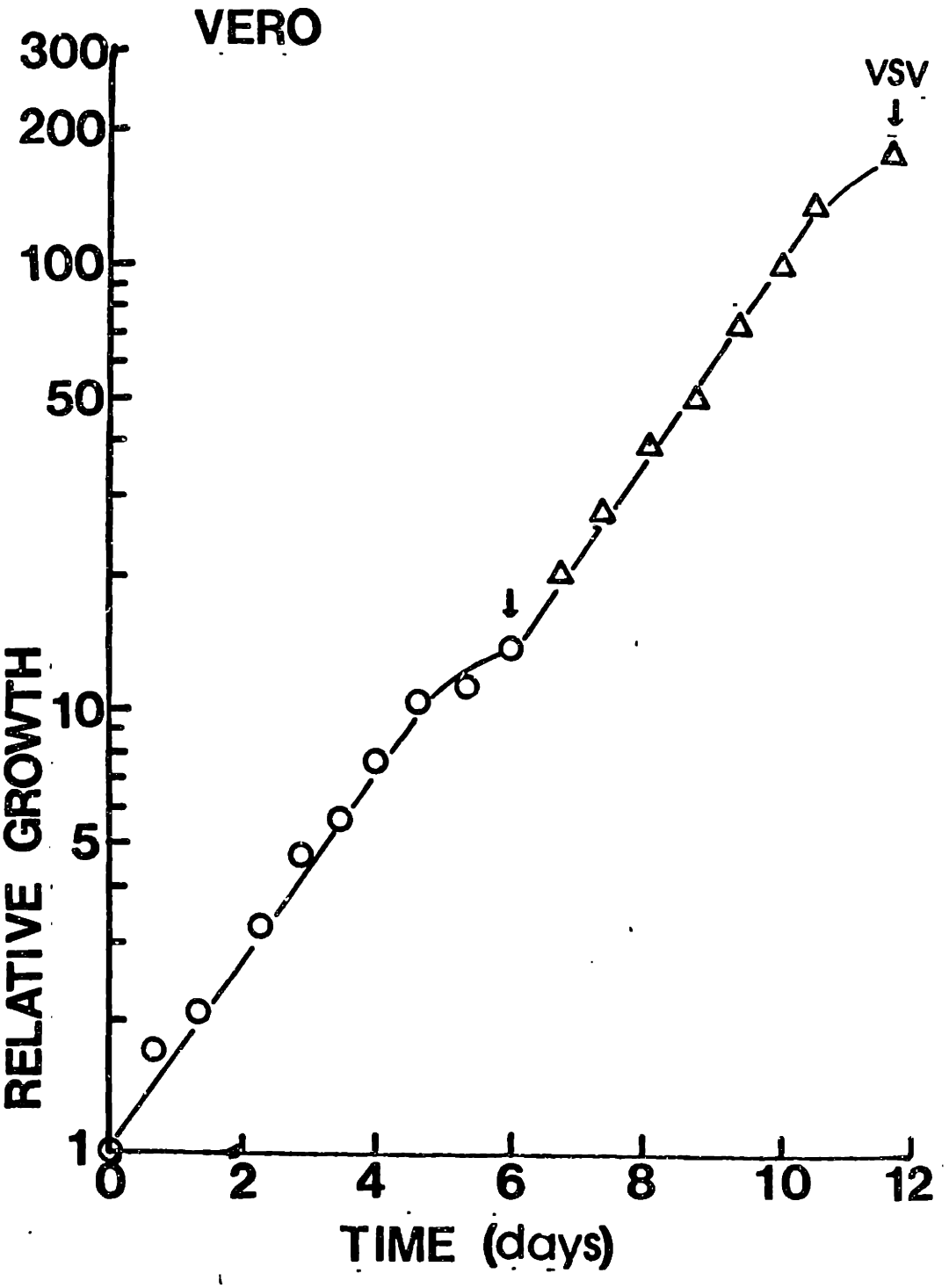
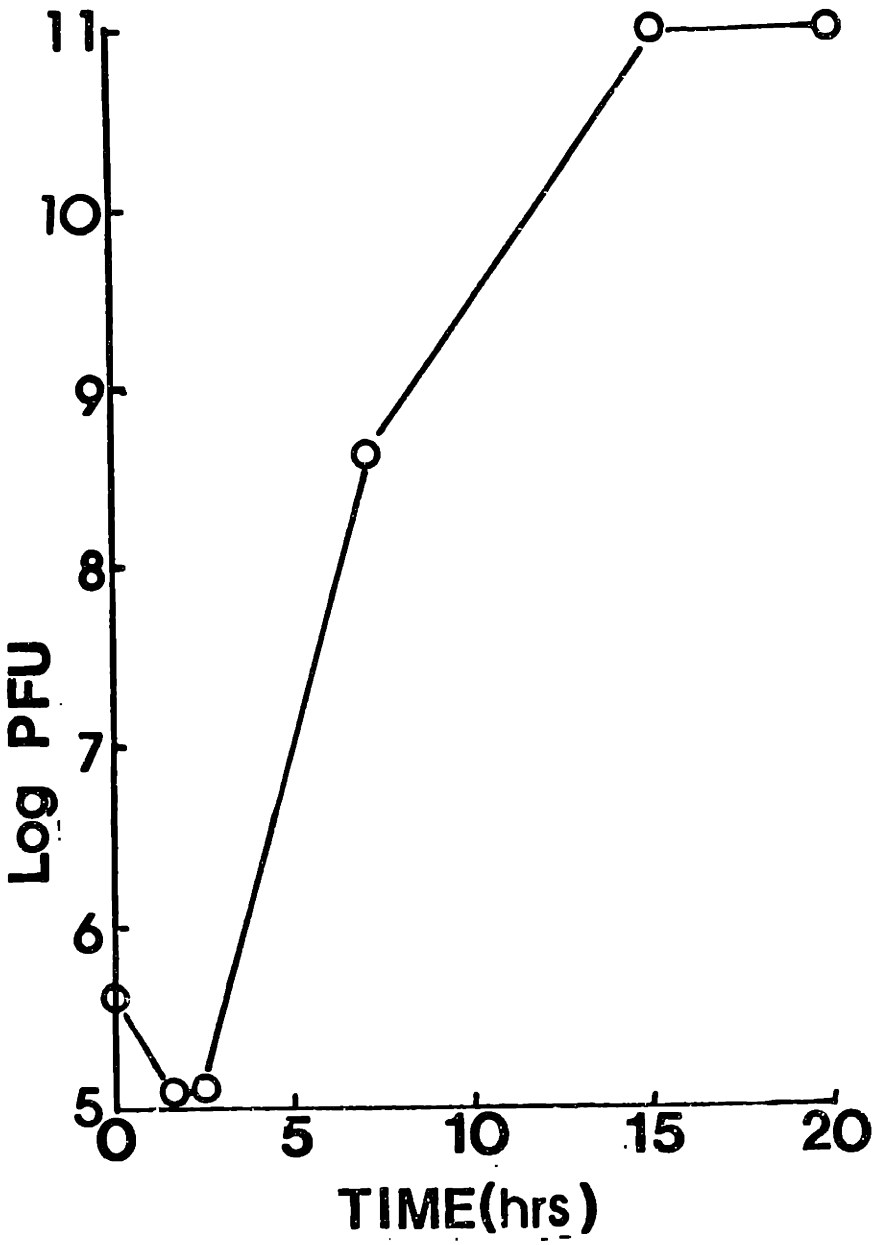

VERO
VSV
300
200
100
50
10
5
1
RELATIVE GROWTH
0
2
4
6
8
10
12
TIME (days)

Figure 2-14. Vesicular Stomatitis Virus Production Using Serially Propagated Vero Cells.


11
10
9
8
7
6
5
Log PFU
0
5
10
15
20
TIME(hrs)

The results presented in this chapter are for specific cell types. The conditions required to detach cells from the substrate are affected by the cell type, the serum concentration and the medium used for the cultivation, and the extent of confluency as well as the length of time cells have been in the confluent stage. For a particular cell type under a set of specific culture conditions, the required trypsin and EDTA concentrations or pH may vary. However, the general principles developed here for the successful serial propagation of cells on microcarriers should be generally applicable to all types of cells.

CHAPTER 3. SELECTION OF MICROCARRIER DIAMETER FOR SERIAL PROPAGATION

3.1 EFFECT OF MICROCARRIER DIAMETER ON OTHER GROWTH PARAMETERS

Anchorage-dependent cells are normally cultivated in a batch mode. In each batch culture a certain number of cell doublings can be achieved. A series of batch cultures of increasing scale is typically used to reach the production stage. The developments described in Chapters one and two allow one to propagate mammalian cells serially on microcarriers in a specified number of propagation steps and a chosen multiplication ratio. The number of propagation steps and the multiplication ratio are interrelated and both are affected by the microcarrier diameter. In this section the effect of microcarrier diameter on the other parameters will be examined.

The following analysis deals with two hypothetical cultures, each containing uniform microcarriers of two different diameters, D_1 and D_2. Two different cases will be considered in this analysis. In the first case the maximum growth extent allowed in a batch culture is held constant and in the second case the final growth extent is maximized. If the maximum growth extent is held constant, the surface area needed for cell growth, A, will be the same for microcarriers of different diameters.

Thus, the number of microcarriers per unit volume will decrease inversely with the square of the diameter as shown in Eqution 25.

$$A_1 = A_2$$

$$N_1D_1^2 = N_2D_2^2$$

$$N_1/N_2 = (D_2/D_1)^2$$

(Eq. 25)

To maintain a specified initial cell distribution on the microcarriers, the average cell number per microcarrier at inoculation, U, should be held constant. The inoculum cell concentration, X, required to give rise to a specified cell distribution is thus inversely proportional to the square of the microcarrier diameter. This is shown in Equation 26.

$$U_1 = U_2$$

$$X_1/N_1 = X_2/N_2$$

$$X_1/X_2 = (D_2/D_1)^2$$

(Eq. 26)

The microcarrier concentration, W (gram/liter), required to maintain a constant surface area increases linearly with the diameter as illustrated by Equation 27.

$$W_1/W_2 = N_1D_1^3/N_2D_2^3 = D_1/D_2$$

(Eq. 27)

The multiplication ratio (mr) is defined as the ratio of the maximum growth extent to the inoculum cell concentration. In this case the maximal growth extent is held constant and the inoculum cell concentration descreases with increasing microcarrier diameter (Eq 26). The multiplication ratio increases with the square of the diameter. This is shown in Eq. 28.

$$mr_1/mr_2 = (D_1/D_2)^2$$

(Eq. 28)

In summary, when the total surface area is held constant, the inoculum concentration needed to achieve the same initial cell distribution decreases with increasing microcarrier diameter. The multiplication ratio achievable in each propagation step increases with the square of the diameter. The number of propagation steps required to reach production scale can thus be reduced with the use of larger microcarriers. However, a concurrent increase in microcarrier concentration is necessary to provide the same surface area. The agitation rate required to keep microcarriers in suspension increases with both the diameter and the concentration of microcarriers. Excessive shear exerted by the impeller at a higher agitation rate causes cell death. Thus, the largest size of microcarriers that can be employed is limited by the maximum shear force that the cell type can withstand.

In choosing the microcarrier diameter, it is more desirable to maximize the microcarrier concentration than to maintain a constant surface area. In this case the microcarrier concentration, W, will be held constant and the effect of changing the diameter under this constraint will be examined. When the microcarrier concentration is held constant, the number of microcarriers per unit volume decreases inversely with the cube of the diameter as shown in Equation 29.

$$W_1 = W_2$$

$$N_1 D_1^3 = N_2 D_2^3$$

$$N_1/N_2 = (D_2/D_1)^3$$

(Eq. 29)

The total surface area available for cell growth decreases linearly with the reciprocal of the diameter as shown in Equation 30.

$$A_1/A_2 = N_1 D_1^2 / N_2 D_2^2 = D_2/D_1$$

(Eq. 30)

Since the maximum growth extent is limited by the available surface area, the growth extent possible is also inversely proportional to the microcarrier diameter.

To achieve a specified initial cell distribution, the mean cell number per microcarrier should be held constant. Thus, the required inoculum concentration decreases inversely with the cube of the diameter. This is shown in Equation 31.

$$U_1 = U_2$$

$$X_1/N_1 = X_2/N_2$$

$$X_1/X_2 = (D_2/D_1)^3$$

(Eq. 31)

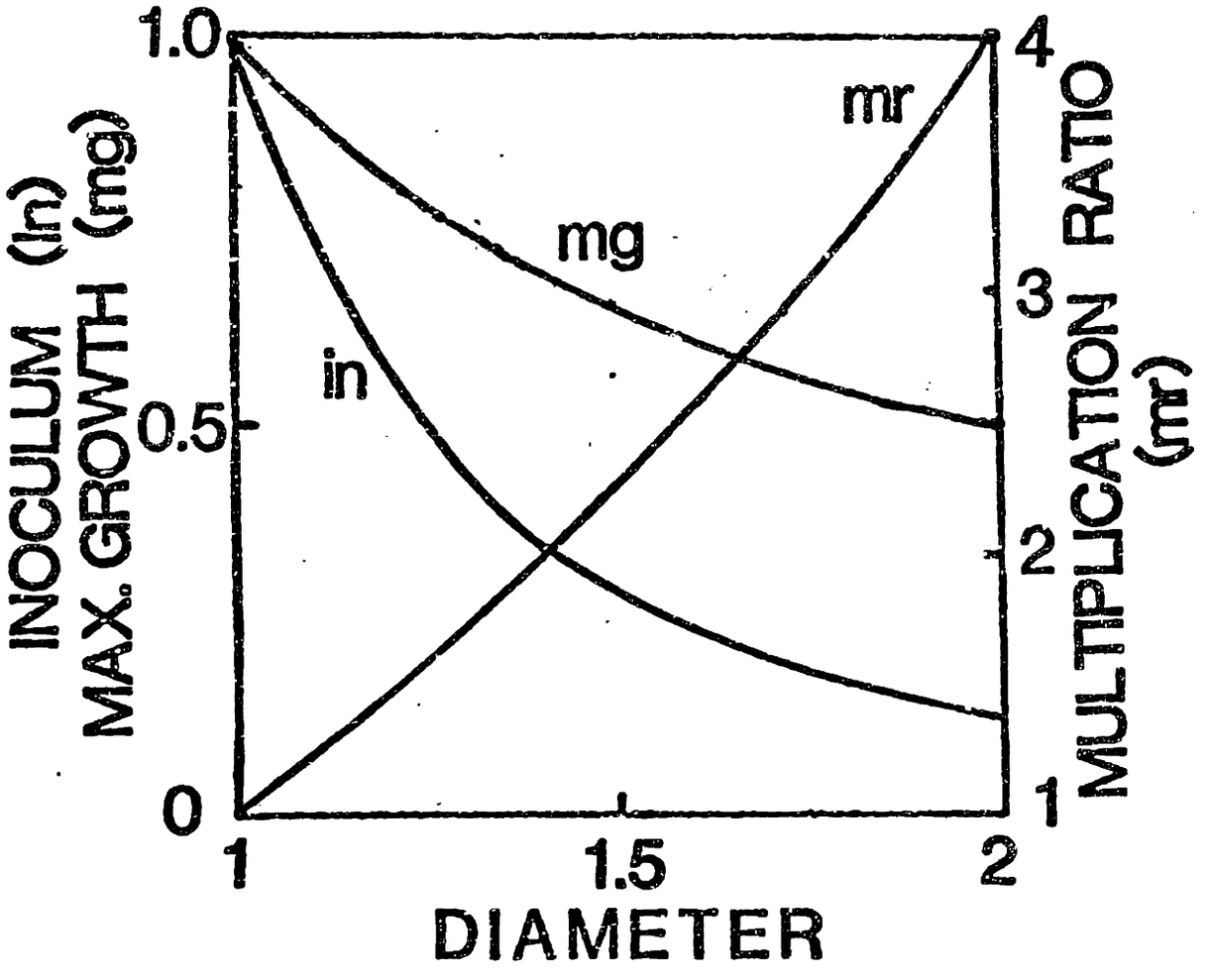
The multiplication ratio, mr, in each stage is again the ratio of the maximal growth extent to the inoculum concentration. It increases with the square of the microcarrier diameter. This is shown in Equation 32.

$$mr_1/mr_2 = (D_1/D_2)^2$$

(Eq. 32)

The relationships between these parameters and microcarrier diameter are shown on Figure 3-1. When the microcarrier concentration is held constant, both the inoculum cell concentration required for a given cell distribution on microcarriers and the maximal extent of growth are decreasing, inverse functions of microcarrier diameter; whereas the achievable multiplication ratio is an increasing function of the diameter. The net increase in cell concentration is the difference between the maximum growth extent and the inoculum cell concentration. The net increase in cell concentration is therefore also affected by the microcarrier diameter. The microcarrier diameter which gives rise to the maximal net increase

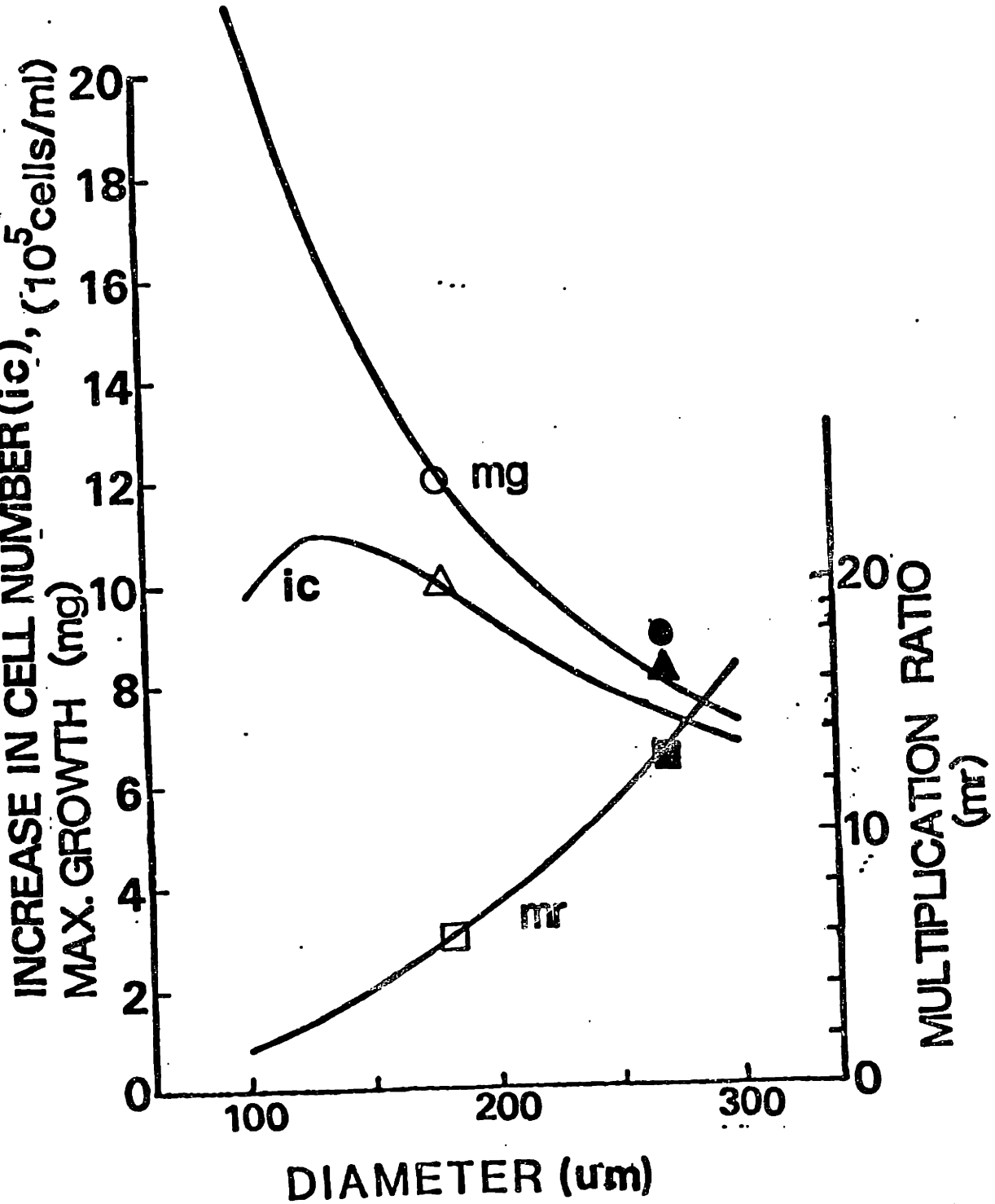
Figure 3-1. Effect of Microcarrier Diameter on Inoculum Cell Concentration, Maximal Cell Growth Extent and Multiplication Ratio.


1.0
0.5
0
INOCULUM (in)
MAX. GROWTH (mg)
mr
mg
in
4
3
2
1
MULTIPLICATION RATIO
(mr)
1
1.5
2
DIAMETER

in cell concentration can be defined once other operating conditions are specified.

Shown in Figure 3-1 are normalized values; the actual magnitudes of these parameters vary with the maximum growth extent of the cell type used and the desired initial cell distribution. Figure 3-2 shows one specific example which was calculated for FS-4 cells with a initial inoculation cell density of eight cells per microcarrier. For purposes of comparison, all microcarriers were assumed to be uniform in size and the microcarrier concentration was 5 g/l. The maximum growth extent is a decreasing, inverse function of the diameter, while the multiplication ratio is an increasing function. The net increase in cell concentration, which is the difference between the maximum growth extent and the inoculum concentration is also shown. It exhibits a maximum at the microcarrier diameter of 130 μ. With microcarriers having a diameter of 130μ, the achievable multiplication ratio is three. An increase in microcarrier diameter improves the multiplication ratio but results in a lower net increase in cell concentration. The experimental results using microcarriers of two different median diameters are also shown in Figure 3-2. Considering that the microcarriers used in the study were not uniform in size, the experimental results fit the calculations quite well. Therefore, for the cultivation of FS-4 cells, maximum gain in cell number and a high multiplication ratio cannot be obtained simultaneously. The two goals have to be reconciled in a microcarrier culture.

Figure 3-2. Effect of Diameter on the Growth Extent, Cell Yield and Multiplication Ratio of FS-4 Cells. Curves are calculated for the inoculation cell density of eight cells per bead. The data for the two bead diameters used in this study are also shown. Microcarrier concentration was 5 g/l. (○,●): maximal growth extent, cells/ml; (□ ,■): multiplication ratio; (△,▲): increase in cell concentration, cells/ml.


INCREASE IN CELL NUMBER (ic), (10^5cells/ml)
MAX. GROWTH (mg)
MULTIPLICATION RATIO (mr)
DIAMETER (um)
mg
ic
mr
0
2
4
6
8
10
12
14
16
18
20
100
200
300

3.2 A GENERALIZED STRATEGY FOR SELECTION OF MICROCARRIER DIAMETER

Although most of the results presented in this thesis were obtained with FS-4 cells, the principles on which this study was based are applicable to other cell types. In determining the optimal operating conditions, the important parameters included the cell distribution at inoculation, the multiplication ratio, the absolute increase in cell number in each propagation step, and the microcarrier diameter. These parameters are all interrelated.

For any cell type, the most desirable cell distribution is the one which allows confluent growth to occur on all microcarriers in the shortest time span. In cases in which no critical number of cells per microcarrier is required, an average cell number per microcarrier of eight to ten is desirable to avoid an excessive time span needed to reach the maximum growth extent. In situations in which both the growth extent and the growth rate are reduced at low inoculum cell concentrations, the critical cell number per microcarrier should be defined. Instead of employing a detailed analysis, a quick estimation of the critical number for cell growth can be performed by determining the final growth extent at different inoculum concentrations. For a first approximation, microcarriers can be assumed to be uniform in diameter. According to the model presented in Chapter one, confluent growth can occur only on microcarriers whose initial cell number is larger than the critical number. For

microcarriers which have not acquired enough cells for growth to occur, the cell number will remain unchanged during the time of interest. The fraction of microcarriers which have not acquired enough cells for growth to occur is described by Equation 15 in Chapter 1.

$$P = \sum_{j=0}^{x_c} \frac{e^{-U} U^j}{j!}$$

(Eq. 15)

The fraction of microcarriers whose initial cell number is larger than the critical number is thus expressed as (1 - P). The final growth extent, X_f, at a given initial average cell number is described by Equation 33.

$$X_f(U) = PX_o + (1 - P)X_{max}$$

(Eq. 33)

In Equation 33 X_o is the inoculum cell concentration, and X_{max} is the maximum cell concentration if all microcarriers are confluent. Under a given set of experimental conditions, the inoculum cell concentration, the maximal extent of growth and the average cell number per microcarrier are all known. The final growth extent can thus be calculated for different critical cell numbers, X_c. Calculated values of the growth extents can be compared to the experimental results obtained at different inoculum concentrations. The accuracy of the estimation is

largely dependent upon the size distribution of microcarriers. Using this approximation method, the critical number for FS-4 cells was estimated to be seven to eight cells per microcarrier, as opposed to six cells as obtained by detailed analysis presented in Chapter 1.

Once the critical cell number per microcarrier required for growth is defined, approaches can be taken to minimize it. A sufficiently high inoculum, i.e. a low enough multiplication ratio, must be used to give rise to a cell distribution in which the nongrowing faction of cells is minimized. This constraint thus sets the upper limit of the multiplication ratio. The other constraint that affects the multiplication ratio is differential cell attachment to new and used microcarriers in serial propagation. A minimum limiting multiplication ratio must be set to reduce the fraction of used microcarriers. This minimum multiplication ratio is affected by the ratio of the cell attachment rate onto new microcarriers to that onto used microcarriers as described in Section 2.4.

After the allowable operating range for the multiplication ratio is chosen, a proper microcarrier diameter can be selected. An optimal microcarrier diameter is the one which gives rise to the highest net increase in cell number as described in Section 3.1. Besides the cell distribution on microcarriers and the multiplication ratio , the maximal growth extent and the net increase in cell number are also affected by the microcarrier diameter. Therefore, a procedure of iteration may be required

for some cell types in order to optimize the microcarrier diameter for a specific process.

CHAPTER 4. STUDIES OF OTHER ENGINEERING PARAMETERS IN MICROCARRIER CULTURE

4.1 EFFECT OF SHEAR FORCE ON CELL GROWTH

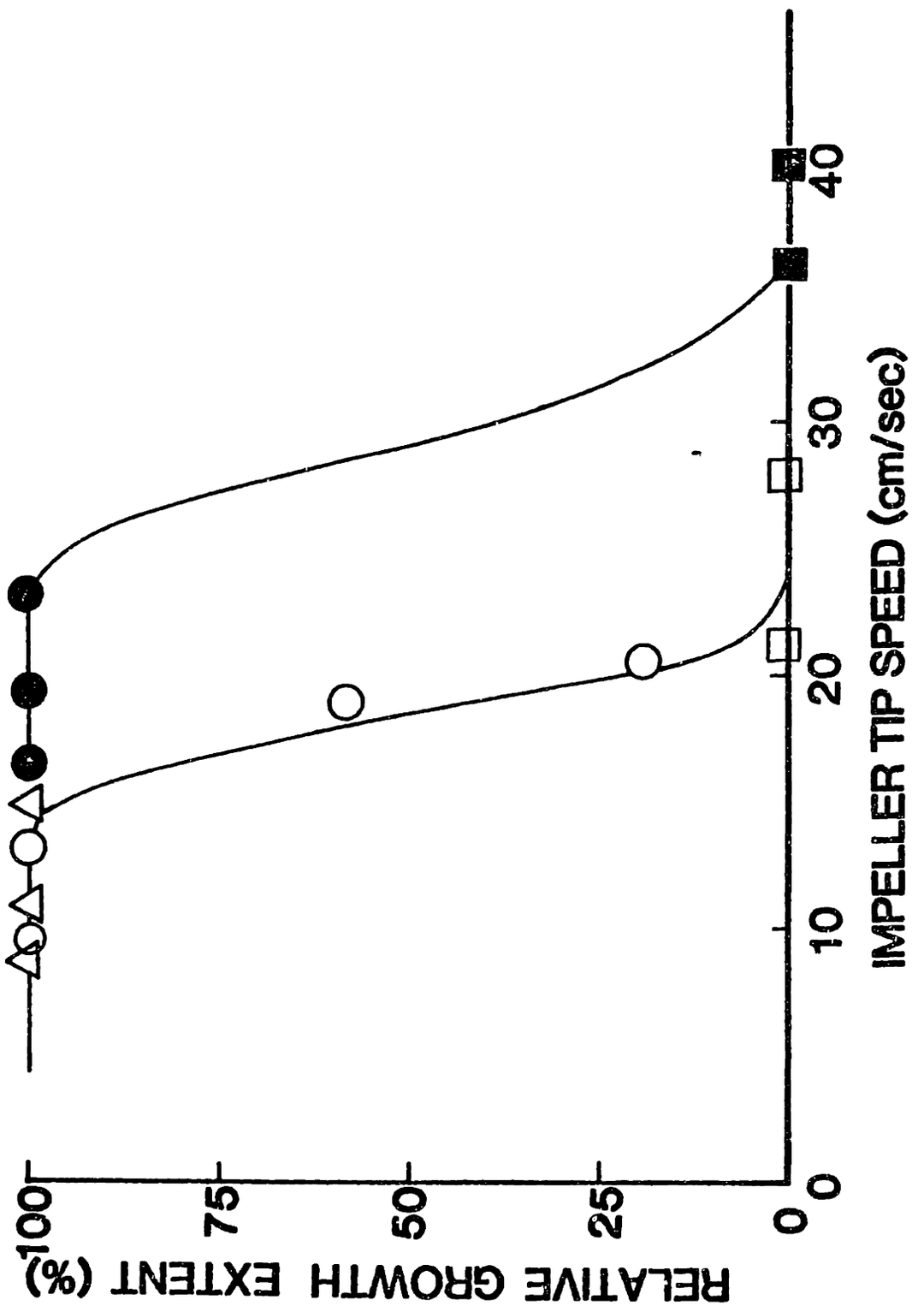
Since the development of microcarriers for the cultivation of anchorage-dependent animal cells, there have been several studies concerning different types of vessels for microcarrier cultures. Among those reported have been fluidized-bed reactors (Hirtenstein et al., 1982) and tank reactors with different mixing mechanisms, such as vibro-mixers (Feder and Tolbert, 1983). However, the mechanically agitated stirred tank reactor is still the type most widely used in microcarrier cultures. The purpose of agitation in microcarrier culture is to suspend microcarriers and achieve fluid mixing, and not to provide mechanical energy for oxygen transfer as in microbial cultures. It has been noted that animal cells are sensitive to shear force exerted by fluid flow (Nevaril et al., 1968; Augenstein et al. , 1971); excessive shear force can lead to cell death. To avoid this detrimental effect of shear force, a low agitation rate is usually used in cell culture.

In order to define the proper agitation conditions for the cultivation of FS-4 cells , an experiment was carried out to assess the effect of shear force in spinner vessels. The spinner vessels used were each equipped with a suspended, teflon-coated,

cylindrical magnetic rod as the impeller. Cells were inoculated into 250 ml vessels each with 100 ml of culture volume, or 2 l vessels each with 1 l of culture volume, containing microcarriers at 5 g/l. An inoculum concentration of 3.5 x 10^5 cells/ml was used for each culture. Impellers of different diameters were used. After inoculation, the minimum agitation required for complete suspension of microcarriers was used in each vessel in order to allow cells to attach and spread onto the microcarriers. Twenty-four hours later, different agitation speeds were then applied to the vessels in order to compare the effect of different agitation conditions on cell growth. The cultures in which normal growth occurred reached confluence in five days. At higher agitation rates, a decreased growth extent was observed. The final growth extent under different agitation conditions is plotted against the impeller tip speed in Figure 4-1. It can be seen that at high impeller tip speeds the observed growth extents were lower. In four cases, the high impeller tip speed resulted in cell death. However, the results in Figure 4-1 also show that the relationship between growth extent and impeller tip speed is different for the two sizes of culture vessels. For example, higher tip speeds could be maintained in the larger vessel before the growth extent was decreased. In order to assess this phenomenon, the results were analyzed further.

It was reported previously by Sinskey et al., 1982) that the growth extent of chick embryo fibroblasts under different shear conditions could be correlated to an

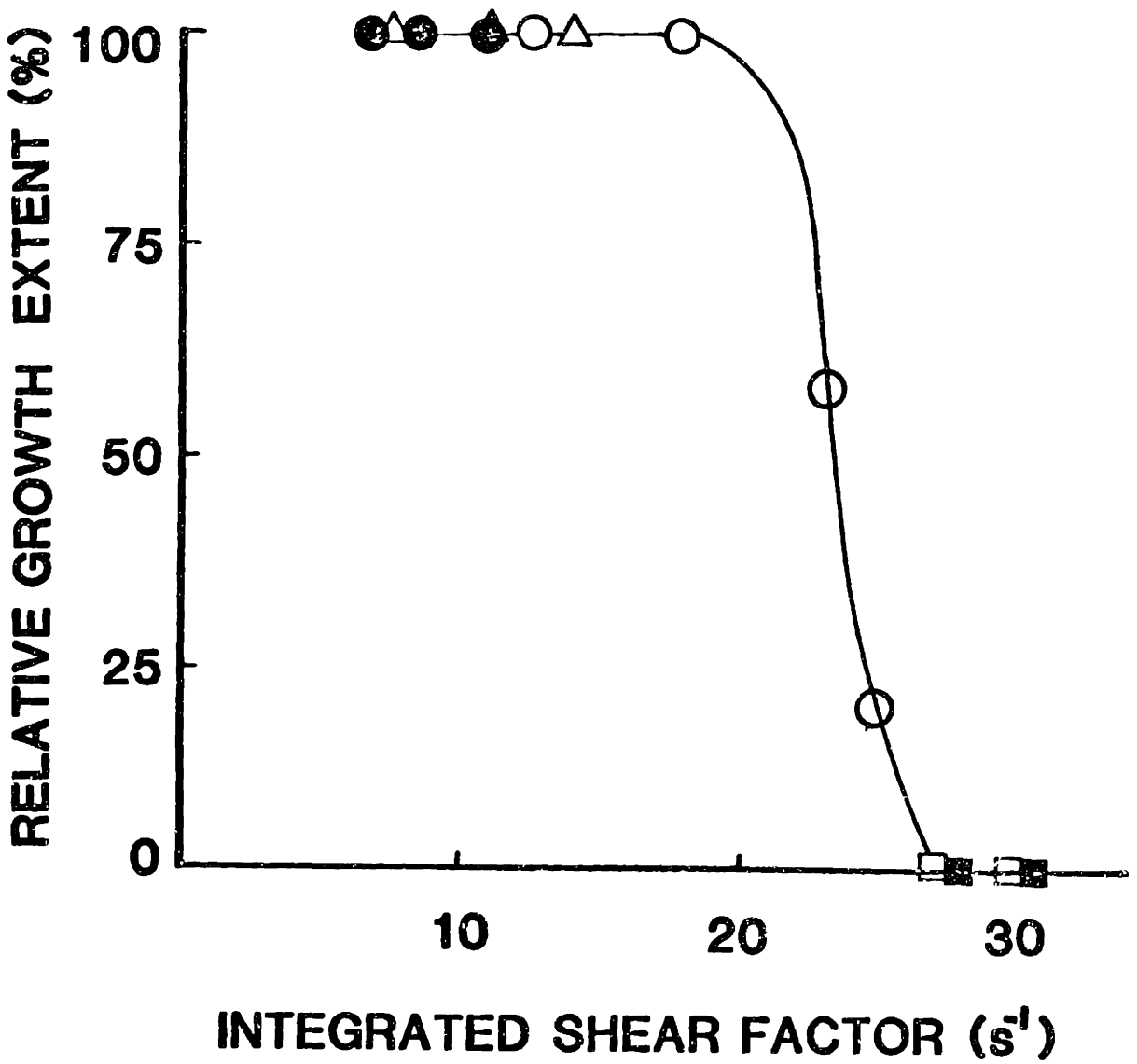
Figure 4-1. Effect of Impeller Tip Speed on the Growth Extent of FS-4. Symbols: 250 ml vessel with 3.2 cm diameter impeller (△), with 4.1 cm diameter impeller (○), with 5.1 cm diameter impeller (□); 2 l vessel with 7.5 cm impeller (●), with 8.5 cm impeller (■).


RELATIVE GROWTH EXTENT (%)
100
75
50
25
0
0
10
20
30
40
IMPELLER TIP SPEED (cm/sec)

integrated shear factor. This integrated shear factor was defined as $2\pi nDi/(Dt-Di)$, where Dt is the vessel diameter, Di is the impeller diameter, and n is the agitation speed. In the integrated shear factor, πnDi represents the impeller tip speed and $(Dt-Di)/2$ the distance between the impeller tip and the wall of the vessel. Since the fluid velocity at the wall is zero, the integrated shear factor is an indication of the magnitude of the velocity gradient in the radial direction along the impeller. The correlation between the growth extent and the integrated shear factor under different agitation conditions was analyzed. There appears to be good correlation throughout the range of experimental conditions tested (Figure 4-2).

The results shown in Figures 4-1 and 4-2 demonstrated that the growth of FS-4 cells on microcarriers is sensitive to the shear force caused by agitation. However, for normal culture conditions, for instance a 4.1 cm diameter impeller in a 250 ml spinner vessel agitated at 60 rpm, the impeller tip speed falls well below the region in which a detrimental effect was observed. In a stirred tank, maximum shear rate correlates with the impeller tip speed, whereas the average shear rate correlates with the agitation rate (Nagata, 1975). As stirred tank reactors are scaled up with geometric similarity, the maximum shear rate, around the impeller tip, goes up, whereas the average shear rate does not increase (Oldshue, 1966; Nagata, 1975). For the growth of FS-4 cells, the important parameter in the assessment of shear force appears to be the integrated shear factor, rather than the impeller tip speed. Under the constraint of geometric

Figure 4-2. Correlation Between the Integrated Shear Factor and the Growth Extent. Symbols are the same as those in Figure 4-1.


RELATIVE GROWTH EXTENT (%)
100
75
50
25
0
10
20
30
INTEGRATED SHEAR FACTOR (s⁻¹)

similarity, Di/(Dt-Di) in the integrated shear factor is constant for reactors of different sizes; thus, the scale sensitive parameter is the impeller speed, n. When the power input per unit volume is held constant, the impeller speed as well as the average shear rate decrease with increasing scale (Nagata, 1975). It has been noted that the power per unit volume requirement for the suspension of solids decreases when the scale is increased (Zwietering, 1958). Under the operating conditions used in this study, the shear force exerted by mechanical agitation has no detrimental effects on the growth of FS-4 cells. Further studies using reactors of different scales are necessary in order to obtain a more reliable correlation for scale-up. Nevertheless, the results presented in this section indicate that excessive shear force is not likely to be a problem in larger scale reactors.

4.2 USE OF SURFACE AERATOR TO IMPROVE OXYGEN TRANSFER

In animal cell culture, the oxygen required for cell growth is typically supplied by surface aeration. As shown in Section 4.1, excessive shear force is detrimental to cells; thus, the agitation speed must be maintained much lower than in microbial fermentations. When air is sparged into the culture vessel, the entrapment and break-up of air bubbles due to agitation is minimal. Thus, the residence time of the air bubbles is short and the interfacial area for oxygen transfer is small. As a

result, the volumetric oxygen transfer coefficient (K_La) is much smaller than in microbial fermentations for a given air space velocity. To achieve a significant improvement in oxygen transfer a high air flow rate would be necessary, especially in small culture vessels in which the residence time of air bubbles is very short. Excessive aeration by sparging, however, causes serious foaming and microcarrier agglomeration, resulting in cell death. The use of air sparging to improve oxygen transfer in a microcarrier cell culture thus presents other serious problems.

In microcarrier culture, oxygen transfer by surface aeration is limited by the low agitation speeds. Under typical agitation conditions for microcarrier culture, little turbulence occurs at the liquid surface; the resistance to mass transfer at the gas-liquid interface is thus high. For cell lines whose specific oxygen consumption rate and confluent cell concentration are sufficiently high, oxygen transfer is the growth limiting factor even in one-liter vessels (Sinskey et al., 1982). A simple method to improve oxygen transfer would be to induce surface turbulence. This could possibly be achieved by the use of a surface aerator. Surface aerators of various types are often used in waste treatment to improve oxygen transfer (Zlokarnik, 1979). Surface aerators were installed in culture vessels to determine their effect on oxygen transfer. The geometry of the surface aerator used in this study is shown in Figure I-1 in the Materials and Methods Section. The oxygen transfer coefficients of the culture vessels were measured as described in Materials and Methods. For purposes of observing

the effect of surface aerators, the oxygen transfer rate was measured in phosphate buffer saline with and without surface aerators. The results are shown in Table 4-1. A greater than four-fold increase in the oxygen transfer rate was attained with a surface aerator in both a one-liter vessel with a 500ml liquid volume and an eight-liter vessel with a 5 l working volume.

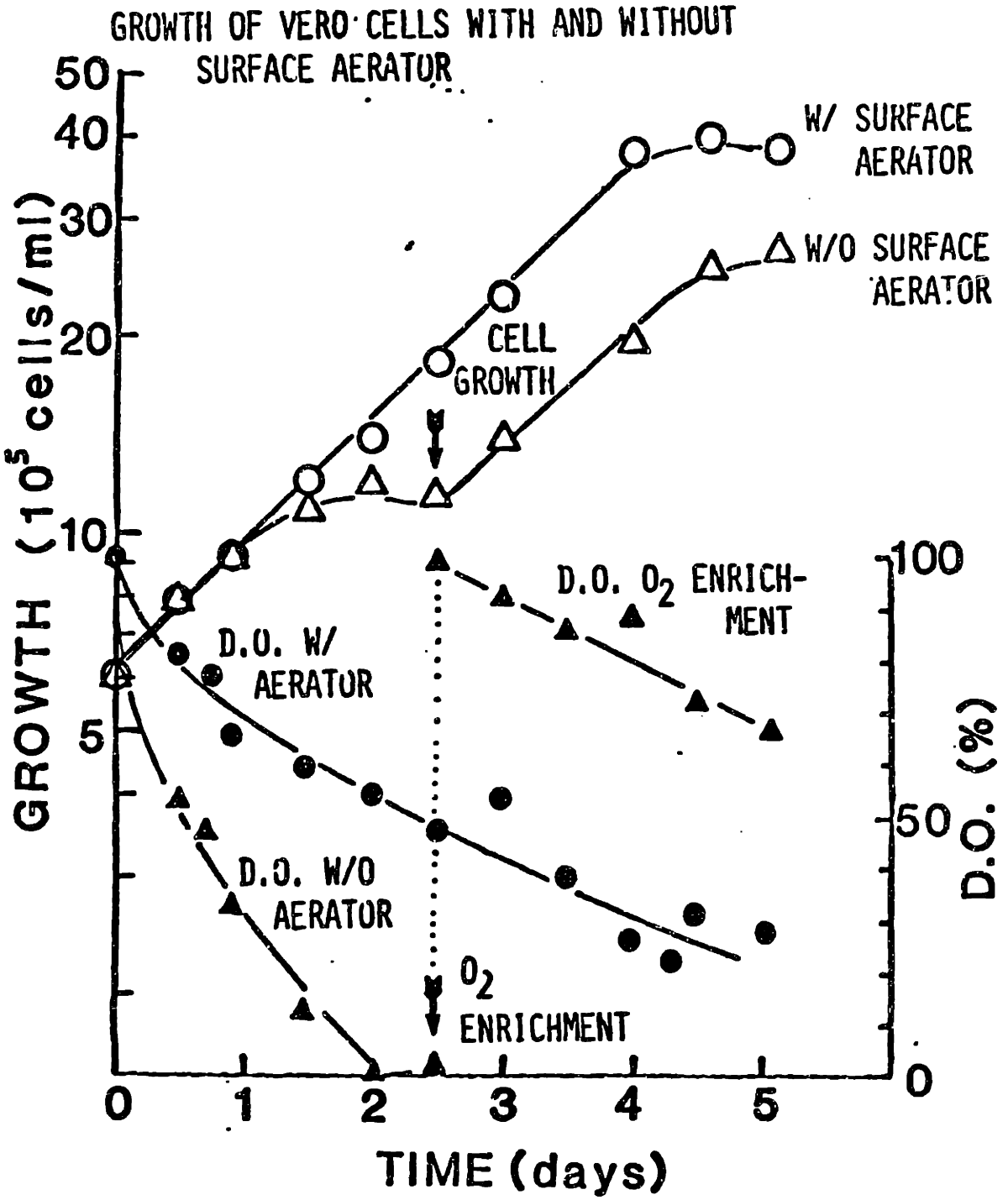
To further evaluate the effectiveness of the surface aerator, Vero cells were inoculated into one liter vessels both with and without a surface aerator at a microcarrier concentration of 5 g/l. The growth medium was DME supplemented with 10% horse serum. After the third day, 50% of the medium was replaced with fresh medium each day. Under such conditions, the confluent cell concentration was typically 3.5 to 4.0 x 10^6 cells/ml. The results are shown in Figure 4-3. Without the surface aerator, the oxygen consumption rate quickly exceeded the supply rate and cell growth was retarded due to oxygen limitation. Exponential growth resumed only after oxygen-enriched air was introduced to the head space to increase the oxygen transfer rate. In the vessel with the surface aerator, the dissolved oxygen concentration decreased at a much slower rate and cells grew exponentially until they approached confluence.

Oxygen transfer is often the growth-limiting factor in animal cell culture. The use of a surface aerator alone will surely not solve this problem for large scale operation; however, on any scale in which surface aeration plays an

Table 4-1. Oxygen Transfer Coefficient in Vessels with and without Surface Aerator.

	Mass Transfer Coefficient (K_L)	
	one liter vessel (500ml medium)	eight liter vessel (5 L medium)
without aerator	6.4 cm/hr	3.3 cm/hr
with aerator	26.2 cm/hr	13.5 cm/hr

Figure 4-3. Effect of the Surface Aerator on the Growth of Vero Cells on Microcarriers. (O): cell growth with surface aerator; (Δ): cell growth without surface aerator; (•): dissolved oxygen level with surface aerator; (▲): dissolved oxygen level without surface aerator. Arrows indicate the use of oxygen enriched air in the vessel without surface aerator.


GROWTH OF VERO CELLS WITH AND WITHOUT SURFACE AERATOR
GROWTH (10^5 cells/ml)
50
40
30
20
10
5
W/ SURFACE AERATOR
W/O SURFACE AERATOR
CELL GROWTH
D.O. O_2 ENRICHMENT
D.O. W/ AERATOR
D.O. W/O AERATOR
O_2 ENRICHMENT
100
50
0
D.O. (%)
0
1
2
3
4
5
TIME (days)

important role in oxygen transfer, a surface aerator will certainly have an enhancement effect.

SUMMARY AND CONCLUSIONS

The studies presented in this thesis have addressed a number of subjects relevant to the large-scale cultivation of mammalian cells on microcarriers. The results and conclusions can be summarized as follows:

I. Inoculum Requirement

1. A minimum inoculum concentration is required for the cultivation of FS-4 cells on microcarriers. A suboptimum inoculum cell concentration results in a decreased growth rate and growth extent.
2. A critical cell number model was proposed and presented mathematically to simulate cell growth on microcarriers. The inoculum requirement can be elucidated with this model. The critical number for growth was determined to be six cells per microcarrier under the culture conditions employed.
3. To reduce the required inoculum cell concentration, it is necessary to reduce the critical cell number as well as to improve the cell distribution on microcarriers. An improved medium for clonal growth was found to reduce the critical number per microcarrier. The cell distribution was improved using microcarriers having a larger diameter. The multiplication ratio for the cultivation of FS-4 cells was increased to fifteen through the

combined optimization of culture medium and microcarrier diameter.

II. Direct Inoculation for Microcarrier Cultures

1. Cells can be detached from microcarriers and used to reinoculate new cultures by treatment with trypsin at an elevated pH (8.4-9.0).
2. Detached cells reattach to used microcarriers at a slower rate than to new microcarriers. This differential attachment results in both poor cell distribution on the microcarriers and poor growth kinetics.
3. To compensate for the effect of differential cell distribution, a high multiplication ratio must be used during serial propagation of cells on microcarriers. This in effect reduces the fraction of used microcarriers.
4. FS-4 cells serially propagated on microcarriers at a high multiplication ratio are fully capable of normal interferon production. Similarly, Vero cells propagated in this fashion are capable of producing vesicular stomatitis virus.

III. The results obtained with FS-4 cells were used to devise a general scheme for the optimization of microcarrier diameter.

IV. Other Engineering Aspects of Microcarrier Culture

1. The effect of agitation on cell growth was characterized. Growth extent under different agitation conditions can be correlated with the integrated shear factor.
2. Oxygen transfer in cell culture was analyzed. A surface aerator can be used to improve the oxygen transfer rate.

RECOMMENDED FUTURE RESEARCH

With the advent of recombinant DNA technology, animal cell culture is becoming more important as a possible method for the production of biologically active substances. Animal cells can be used to produce proteins which require post-translational modifications which cannot be processed properly in bacterial systems. The studies presented in this thesis should help to pave the way toward large-scale operation in microcarrier culture. However, further studies are necessary before such large scale operations can be implemented.

One of the pressing problems in microcarrier culture is oxygen transfer. In animal cell culture processes, oxygen is typically transferred through surface aeration. The oxygen transfer coefficient (k_L) for surface aeration is on the order of 0.005 cm/s. The specific oxygen consumption rate of mammalian cells ranges from 4 to 15 x 10^{-11} mMoles/cell-hr. The maximum cell concentration, for cell lines such as Vero cells at 15 g/l of microcarriers, is about 1 x 10^7 cells/ml. The oxygen consumption rate in a culture can therefore be as high as 1.5 mM/hr. For a one-hundred-liter vessel with a liquid height equal to the diameter, the specific surface area is 0.02 cm^{-1}. The solubility of oxygen at atmospheric pressure is about 0.18 mM. Thus, the oxygen transfer rate will be less than 0.06 mM/hr which will be significantly lower than the demand (1.5 mM/hr). Under

these conditions, oxygen transfer can severely limit the capability of a cell culture bioreactor.

This problem has been alleviated in medium-sized reactors by the use of silicone tubing for oxygen transfer. However, the use of a silicone tubing system for oxygen transfer could be cumbersome in large-scale operation. Improving oxygen transfer by circulating the medium through an external oxygenating vessel is an alternative but poses another problem. Because of the low solubility of oxygen in the culture medium, a very high circulation rate would be needed. Using the figures presented above, as much as nine culture volumes per hour are required for the recirculation rate.

To improve oxygen transfer in microcarrier culture, direct sparging would probably be the most effective method. However, other problems involved with direct sparging must be overcome. Past failures in supplying oxygen by sparging should not be interpreted as proof of its impossibility. Foaming caused by sparging can possibly be suppressed by proper selection of an anti-foaming agent. Care should be taken to assess the effect of sparging on cell growth. The terminal rising velocity of air bubbles is much higher than the terminal settling velocity of microcarriers; the shear generated by air bubbles may well be detrimental to cells if microcarriers surrounding the air bubbles are entrained by them.

A better understanding of the effect of the dissolved oxygen concentration on cell growth and metabolism is also necessary in order to quantify the oxygen supply needed. For

some cell types, the maximum growth rate can often be maintained even at a relatively low levels of dissolved oxygen concentration. Controlling dissolved oxygen at an arbitrarily high level by continual sparging could result in excessive aeration which might not be desirable.

Another important area which should be addressed is the strategy of medium design for microcarrier culture. A typical animal cell culture medium is a balanced salt solution containing sugar, amino acids, and other nutrients. However, in order for animal cells to grow, it is necessary to supplement the medium with 5-10% calf or horse serum. The relative cost of serum for animal cell culture is staggering; besides the cost, serum complicates the product separation process. For the manufacture of products for medical use, serum may also introduce contaminants into the manufacturing process. Such contaminants include mycoplasma and animal viruses (Hopps, 1974). Progress in recent years has made the growth of some types of cells in serum-free culture possible (Bettger et al., 1981, McKeehan et al., 1981; Mather et al., 1981). However, most such studies have dealt only with cell growth at very low density. Many media developed in those studies were designed primarily for clonal growth, in which the changes in nutrient concentrations are negligible throughout the growth period. The cell concentration in microcarrier culture is often one to two orders of magnitude greater than in conventional cell culture. Therefore the media designed for clonal growth are not necessarily directly applicable to microcarrier culture. To benefit from recent

advances in medium development for serum-free growth, it is necessary to examine the nutritional growth requirements from a kinetic point of view.

Cell culture medium provides cells not only with nutrients but also provides a balanced salt concentration and the optimal osmotic pressure. In microbial fermentations, nutrient supply and pH control are often achieved by intermittent feeding. In animal cell culture, however, such feeding may result in a change of osmolarity, thus impeding cell growth. The media presently used in microcarrier culture were all developed for cell growth on conventional cultivation apparatus. Both the nutrient concentrations and the buffer capacity are not sufficient to support the high metabolic rates of cell growth on microcarriers. In order to sustain cell growth, the medium is typically withdrawn periodically, either completely or partially, and replaced with fresh medium. Another mode of medium change is often called perfusion. In a perfused culture, microcarriers are retained in the reactor, whereas the liquid phase is changed continuously. By and large, the important parameters in devising a medium exchange scheme, such as the interval between medium changes and the fraction of the total medium volume to be changed each time, or the flow rate in the case of perfusion, have been determined by trial and error. There has not been a systematic kinetic study to develop guidelines for medium design and medium exchange for mass cell culture. Such guidelines are a prerequisite to any meaningful medium development for serum-free culture.

One of the critical factors in medium design is certainly the optimization of nutrient concentrations. The concentrations of nutrients affect not only the growth of cells but also their metabolism. For instance, the glucose concentration in the culture seems to affect the proportion of glucose which is completely oxidized to that converted to lactate (Imamura et al. , 1982). Controlling glucose at a low concentration by intermittent feeding can possibly reduce lactate formation, as was suggested by Fleischaker (1982). Another nutrient whose concentration may require careful control is glutamine. Excessive glutamine may give rise to ammonia accumulation, thus resulting in growth inhibition. The concentration of serum, or the growth factors in the case of serum-free culture, is usually the growth-rate-limiting factor in a culture medium. To maximize the residence time of serum, it is desirable to change medium in a batch fashion. However, to minimize the concentration of growth inhibitory metabolites, it is probably beneficial to operate in a continuous mode. Therefore, the constraints imposed on the system include the acceptable concentration ranges of nutrients, the kinetic relationships, and the acceptable operating ranges of osmolality and pH, as well as the optimal mode of operation (eg. intermittent vs. continuous medium changes). With the large number of variables involved, it is impossible to exhaustively examine all possible combinations by trial and error to define the optimal conditions. A rational design of the medium for microcarrier culture requires the construction of a kinetic model involving all of these

constraints. The optimal conditions could be solved either analytically or numerically.

The third area requiring further study is the use of microcarrier culture as a biocatalytic reactor for the production of biologicals over an extended period. Most adult animal tissues are non-multiplying cells. Many cell types can be maintained on a conventional surface in a non-dividing state over long periods of time (e.g. months). It is conceivable that cells on microcarriers could retain viability and productivity for long periods under properly controlled conditions. The effect of serum concentration, in particular, should be investigated. Serum contains mitogens which stimulate cell growth. The omission of some mitogens from the culture medium results in arrest of cell growth without affecting cell viability (McKeehan et al., 1981). It is thus possible that,with careful control of the concentrations and the supply rates of mitogens and other nutrients to meet the requirement of cell maintenance, one could diminish cell growth without sacrificing viability and productivity.

APPENDIX. FIBRONECTIN PRODUCTION BY FS-4 CELLS CULTIVATED ON MICROCARRIERS

At the beginning of this thesis research, attention was focused on the production of fibronectin. Fibronectin refers to a group of closely related, large molecular weight glycoproteins present in plasma or on the surface of many cell types (Yamada and Olden, 1978). Although cell surface fibronectin and plasma fibronectin are not identical (Yamada and Kennedy, 1979), they have similar molecular weight, structure, and immunological properties, as well as biological activities (Mosher, 1980; Hynes et al., 1979). Fibronectin can mediate the attachment of cells to collagen (Pearlstein, 1976). The cellular fibronectin is a major component of the cell matrix. The amount of cell surface fibronectin is usually reduced in transformed cells (Chen et al., 1976). Growth of transformed cells exhibits a relatively random pattern. The addition of fibronectin to the culture can restore more normal social behavior; the growth pattern tends to revert to the aligned parallel arrays observed in normal cells.

Fibronectin has been found to stimulate phagocytosis of gelatin-coated colloids by macrophages in vitro (Gudewicz et al. 1980). It is thought to be the humoral recognition factor for phagocytosis by macrophages of the reticuloendothelial system (Blumenstock et al. , 1978). Phagocytosis is the process by which circulating particles, such as bacteria, damaged cells, and fibrin aggregates, are bound to and endocytosed by phagocytotic

cells. The concentration of plasma fibronectin in patients decreases following major surgery or trauma (Scovill et al., 1976). The reversal of fibronectin deficiency by the infusion of fibronectin-rich cryoprecipitate has resulted in improvement in recovery (Scovill et al., 1979). Fibronectin is potentially useful for trauma therapy (Saba et al. , 1978). Fibronectin production was considered then as a model system for the study of product formation by cultured cells. It is synthesized by cells throughout growth, while other products, e.g. viruses and interferons, need to be induced and their production is often restricted to a specific growth stage. To study the feasibility of fibronectin production for potential trauma therapy by human fibroblasts cultivated on microcarriers, a biological assay for fibronectin is necessary. Such a biological assay should reflect the enhancement effect of fibronectin on phagocytosis in vivo. However, such a reliable biological assay was not available. The liver slice assay used in previous work has been shown to reflect particle binding rather than phagocytosis (Molnar et al., 1979). After preliminary studies, it was realized that a major effort would have to be focused on the development of a reliable biological assay if fibronectin production were to be used as a model system. Since the objective of this thesis research was to investigate the engineering parameters important to microcarrier culture, it was decided that the research should focus on the pressing problems of microcarrier culture rather than on fibronectin production.

In the preliminary study of fibronectin production, fibronectin was purified from human plasma. The purified fibronectin was used to develop a radioimmunoassay for fibronectin. The kinetics of fibronectin production by FS-4 cells were also studied. Although these studies were not directly related to the main objectives of this thesis, it was felt that the results should be included for completeness.

PURIFICATION OF FIBRONECTIN

Fibronectin was purified from human plasma or cell culture supernatants by gelatin-Sepharose 4B affinity chromatography (Engvall and Ruoslahti, 1977). Ten ml of gelatin-Sepharose 4B were used for each 50 ml of human plasma. The gelatin-Sepharose 4B mixture was incubated on a shaker at room temperature for two hours to allow fibronectin to bind to the Sepharose beads. A rotating speed of about 50 rpm was used during the incubation period. After incubation the gelatin-Sepharose 4B beads were recovered by centrifugation at 500xg and washed twice with 10 volumes of PBS. The washed beads were loaded onto a chromatographic column for further washing with PBS. Fibronectin was eluted with 4 M urea in PBS. The protein concentration was monitored by the absorbance at 280 nm. The fraction with the

maximum absorbance was dialyzed against 20 mM CAPS (cyclohexylaminopropane sulfonic acid) buffer (pH 8.8) and stored at 4 °C until use.

The fibronectin purified by gelatin affinity chromatography was applied to SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed using 5% or 9% gels as described by Laemmli (1970). Samples were reduced with 0.1% 2-mercaptoethanol and boiled for 2 min before being applied to the gel. The apparent molecular weight of the purified fibronectin was 230,000 as judged from the 5% gel. The molecular weight standards used in the gel electrophoresis were myosine, β -galactosidase, phosphorylase B, bovine serum albumin and ovalbumin. The 9% gel showed little low-molecular weight contaminant present in the purified fibronectin.

The identity of the purified fibronectin was further verified by immunoelectrophoresis. Immunoelectrophoresis was performed in 1% agarose dissolved in 0.05M sodium barbital buffer (pH 8.8). The gel was cast on GelBond film (Marine Products Co.). The thickness of the agarose gel was 1.5 mm. The purified fibronectin was electrophoresed at 7 volts/cm for 1 hr. After electrophoresis, anti-fibronectin serum was added into a trough in the agarose gel. The gel was incubated at room temperature for 16 hours to allow for the formation of immunoprecipitin of fibronectin and antiserum. After the incubation period, the unreacted proteins were allowed to diffuse out for 16 hr in 0.9% NaCl solution and then for 1 hr in distilled water. The agarose gel on GelBond film was then pressed and air dried to a thin film

and stained with amino black 10B. No cross-reacting contaminant was detected in the fibronectin preparation purified by the gelatin-affinity chromatography.

ASSAY OF FIBRONECTIN

To develop an assay to measure the fibronectin concentration in the cell culture supernatant, an electroimmunoassay was first tested. The electroimmunoassay or rocket immunoassay (Laurell, 1966) was performed using 1% agarose gels. Agarose was dissolved in 0.05M sodium barbital buffer (pH 8.8) by heating in a boiling water bath. The agarose solution was cooled to 60 °C before antifibronectin serum was added to yield an appropriate final antiserum concentration. The antiserum-containing warm agarose solution was then pipetted onto GelBond film (8 cm wide, the length varied with the number of sample wells) to give a gel thickness of 1.8 mm. Sample wells 2 mm in diameter were prepared about 1 cm apart from each other. 10 μl each of samples previously diluted to appropriate concentrations were added to each well. The samples were then electrophoresed towards the anode at a voltage of 7 volts/cm at 4 °C for 24 hr. After electrophoresis, the gel was rinsed, dried, and stained as described above for immunoelectrophoretic gels.

To perform the radioimmunoassay the fibronectin purified by gelatin affinity chromatography was used as the standard. Both the standard and the samples of culture supernatant were diluted with fresh culture medium. To each tube, 8,000 cpm of labelled I^{125}-fibronectin were added. This was followed by the addition of competing antigen (standard or sample) and antiserum. The reaction mixture was incubated at 4 °C for 18 hr. Normal rabbit serum and goat anti-rabbit γ-globulin serum were then added to facilitate the formation of immunoprecipitate. The reaction mixture was further incubated for 24 hr. The immunocomplex was precipitated by centrifugation at 2,000x g for 15 min. The supernatant was withdrawn carefully and discarded. The radioactivity in the pellet was counted in a gamma counter.

To determine the proper antiserum dilution for radioimmunoassay, antiserum at different dilutions was added to each tube containing 8000 cpm of labelled fibronectin. The amount of labelled fibronectin precipitated with the immunocomplex varies with the antiserum concentration. The antiserum dilution curve thus constructed is shown as Figure A1. The dilution of antiserum capable of binding 50% of the I^{125}-fibronectin was used for the subsequent competition assay. Shown in Figure A2 are the competition curves typically obtained in this assay. Purified fibronectin at different concentrations was used to construct a standard curve. At least 10 fold linear ranges were obtained with fibronectin concentrations ranging from 20 μg to 2 μg per milliliter. The standard curve and competition curve obtained with the FS-4 cell culture

Figure A1. Binding of I^{125}-Fibronectin At Different Anti-fibronectin Serum Dilutions.

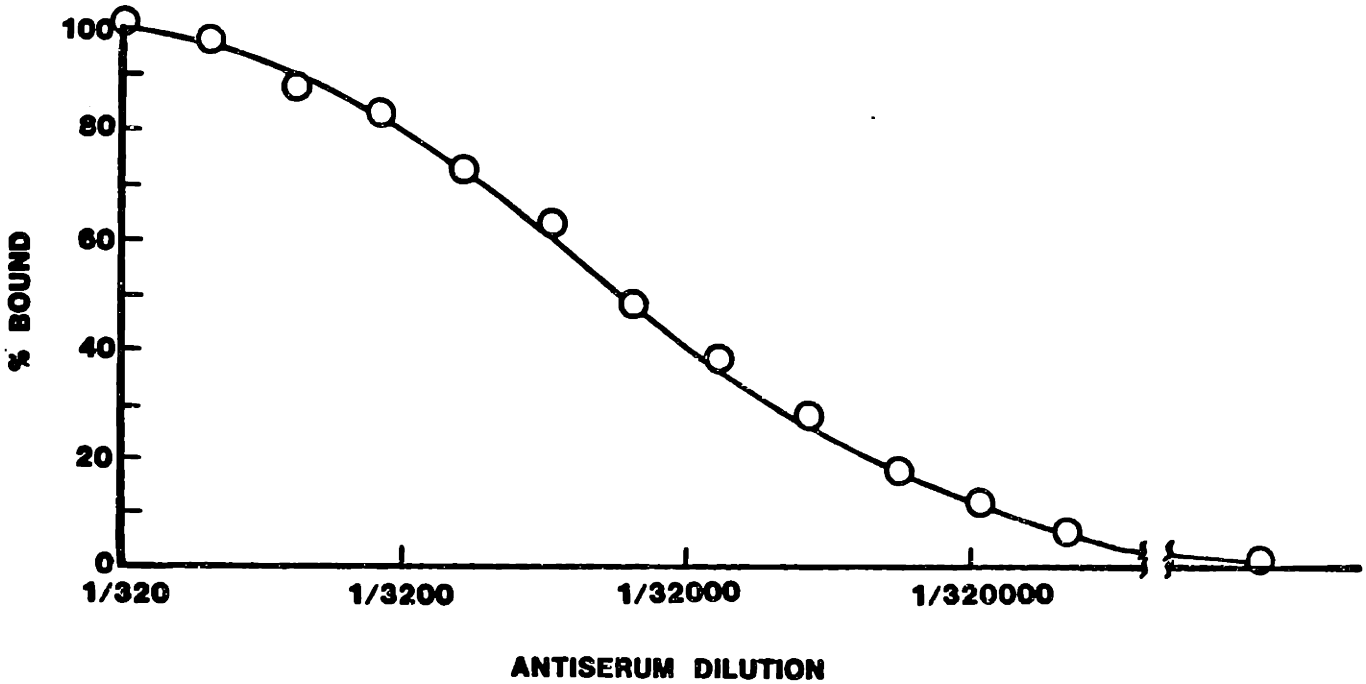
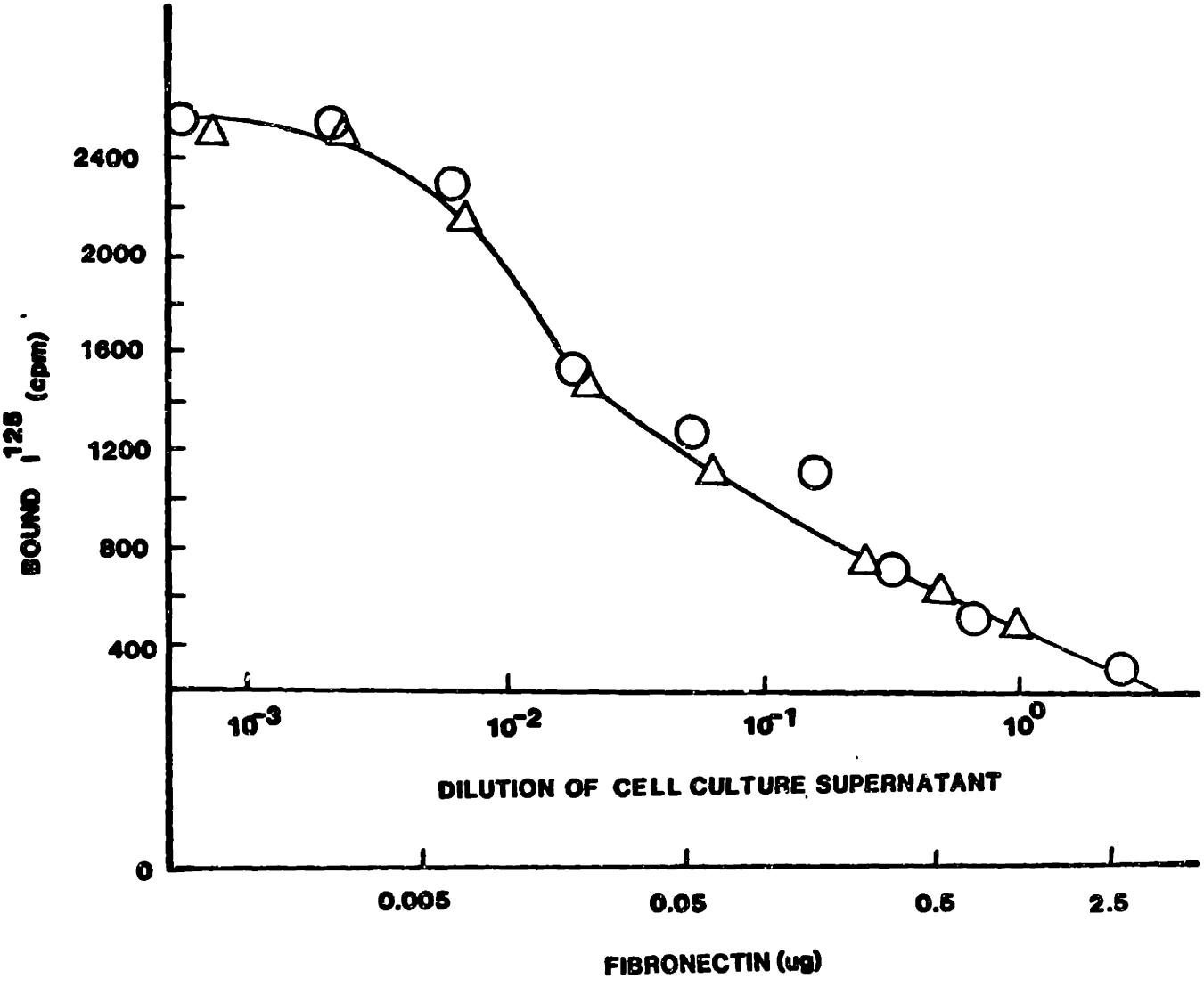

100
80
60
40
20
0
% BOUND
1/320
1/3200
1/32000
1/320000
ANTISERUM DILUTION

Figure A2. Competition Curves for Radioimmunoassay of Fibronectin. (○):standard, purified human plasma fibronectin; (△) a sample of culture supernatant from FS-4 cells grown on microcarriers.

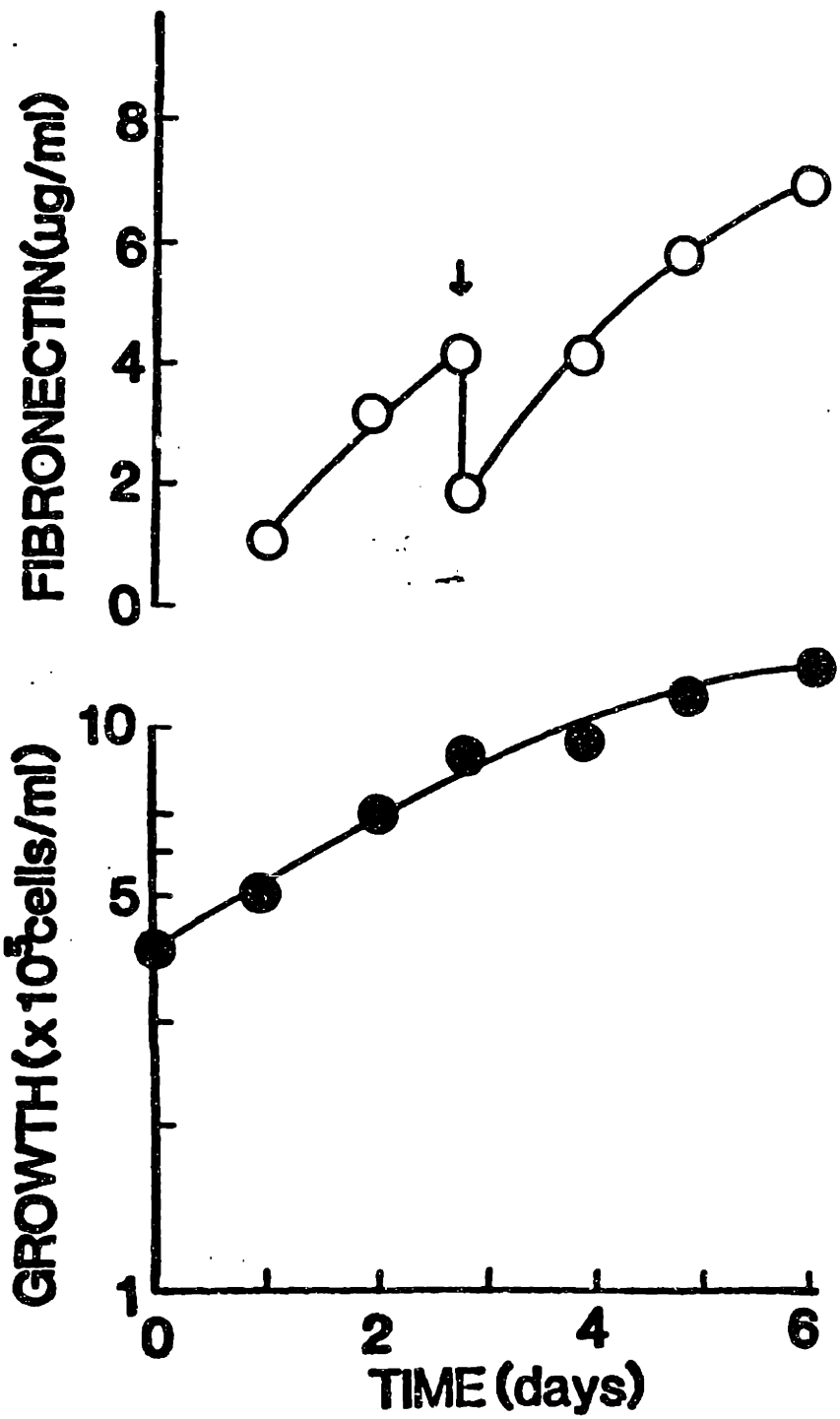

BOUND I^{125} (cpm)
2400
2000
1600
1200
800
400
0
10^{-3}
10^{-2}
10^{-1}
10^{0}
DILUTION OF CELL CULTURE SUPERNATANT
0.005
0.05
0.5
2.5
FIBRONECTIN (ug)

supernatant were superimposable, indicating an immunoidentity of the fibronectin in the supernatant and in the standard. The fibronectin concentration in the culture medium could thus be obtained by comparing to the standard curve.

FIBRONECTIN PRODUCTION BY FS-4 CELLS IN MICROCARRIER CULTURE

Using the radioimmunoassay the kinetics of fibronectin production by FS-4 cells grown on microcarriers were determined and are shown in Figure A3. Half of the medium was changed on the third day of culture. Fibronectin concentrations increased with the increase in cell number, reaching 7 μg/ml on the sixth day with a final cell density of 1.2×10^6 cells/ml. Since a reliable biological assay of the fibronectin-dependent phagocytosis activity was not available, the subsequent focus of research was directed to other, more pressing problems of microcarrier culture, as presented in the Results and Discussion Section.

Figure A3. Kinetics of Growth and Fibronectin Production of FS-4 Cells in Microcarrier culture. (●):cell concentration; (o): fibronectin concentration. The microcarrier concentration was 5 g/l. 50% of the medium was changed on the third day as indicated by (↓).


FIBRONECTIN(ug/ml)
0
2
4
6
8
GROWTH (x10⁵cells/ml)
1
5
10
0
2
4
6
TIME (days)

NOTATIONS

D	the diameter of microcarrier
f_1	the fraction of beads having diameter D_1
f_1	the fraction of used beads in a mixture of new and used ones.
k_1, k_2	rate constant of cell attachment to old and new beads respectively
N	the total number of beads per unit volume, beads/ml
U(D)	the mean of cell number per bead at inoculation for the group of beads with diameter D, cells/bead
X	concentration of cells, cells/ml
X_0	total cell concentration at inoculation, cells/ml
X_1, X_2	cells attached to used and new beads respectively, cells/ml
x	the extent of cell growth on each individual bead, cells/bead
$x_{max}(D)$	confluent cell density on each individual bead, cells/bead
$x_c(D)$	the critical cell number for beads with diameter D, cells/bead
μ	the specific growth rate, hr^{-1}
mr	multiplication ratio, maximum extent of growth/inoculum concentration

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BIOPROCESS SCALE-UP
WORKSHOP/SYMPOSIUM

December 12-15, 1983

Battelle's Columbus Laboratories
505 King Avenue
Columbus, Ohio 43201

Organizing Committee (all from Battelle)

David J. Fink, Co-PI and Workshop Coordinator, (614) 424-7619
Billy R. Allen, Co-PI and Symposium Coordinator, (614) 424-5351
Linda M. Curran, Administrative Coordinator, (614) 424-4743
J. E. Flinn, E. S. Lipinsky, J. H. Litchfield, C. I. Gibson, Advisors

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